POSTHARVEST LEAF BLACKENING IN

Protea neriifolia R. Br.

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

HORTICULTURE

May 1993

BY

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ACKNOWLEDGEMENTS

I would like to thank Dr. Robert E. Paull, my adviser, for his encouragement, stimulation and inspiring advice during the course of this research. His moral support, academic advice, and finance assistant are greatly appreciated. Without his many efforts and criticism, this work could not be finished.

I would also like to thank my committee member, Dr. Richard A. Criley, Dr. Philip E. Parvin, Dr. Douglas J. C. Friend, and Dr. Chung-Shih Tang, for their kindly suggestions and constructive comments on this research work.

I am very grateful to Mr. Roy Tanaka, the farm manager of Maui Branch Station, for providing me with countless shipments of plant materials.

Many thanks to my laboratory mates: Dr. Nancy J. Chen, Gail Uruu, Theodore Goo, Yunxia Qui-O'Malley, Achie and Boots Reyes, and Noreen Endo, with whom everyday was fun, joyful, and unforgettable. Their technical help are also deeply appreciated. Their friendship has become an important part of my life.

My parents and sisters in the People's Republic of China, also provided very important moral supporter.

Finally, from the bottom of my heart, the thanks go to my dear husband Lau Gong. His love, sacrifices and support made my dreams come true. It will never be forgotten.

ABSTRACT

Cut flowers of *Protea* spp. have 20 to 30 leaves attached to their woody stem. The leaves of some species usually turn black within 4 to 8 days after harvest. *Protea neriifolia* R. Br., an important cut flower in the Protea industry, is a species highly susceptible to this problem, and was used in this study of leaf blackening.

Different postharvest stresses lead to different leaf symptoms. Browning and drying of leaves detached from the flower stem, starting from the midrib, was observed under water stress conditions. This leaf browning occurred after 3 days without water supply, following 30% loss of fresh weight. Complete browning occurred over the next 2 days, when the leaf had lost 50% of its fresh weight.

A marginal leaf blackening spreading inward, as well as black spots on the leaf surface were observed when individual leaves were held in the dark. Leaf exudate collected from 10% to 30% blackened leaves in the dark showed a 3 to 5 times higher ion concentration than healthy green leaves, indicating cellular membrane damage and leakage of cell contents. Soluble polyphenol oxidase (PPO) activity also increased in the dark, due probably to the disruption of the chloroplast membrane.

Protea neriifolia inflorescence was 2/3 of the total stem fresh weight and significantly influenced leaf blackening. Flower head removal or girdling of the stem immediately below the flower head significantly delayed leaf blackening. Flowers at five stages of flower opening were characterized for flower head diameter, fresh and dry weight, respiration and nectar production. Flower head diameter increased 2.5

fold from 2.7 cm at stage 1 (very tight bud) to 7.0 cm at stage 5 (bracts reflexed). A parallel increase occurred in flower head fresh and dry weight during opening. Nectar production began at stage 4 (open, cylindrical flower). As the flower opened, the rate of nectar production increased from 2.7 ml to 9.8 ml per flower. Sugar content in the nectar varied from 15% to 23.5%. When sucrose-^{14}C was applied to a flower stem leaf for 24 hr, more than 50% of the radioactivity was found in the nectar. The results suggest that flower head growth and nectar production make the flower a strong sink for available carbohydrates. Carbohydrate withdrawal from the leaves remaining on a cut flower stem may lead to a carbohydrate depletion in the leaf, disruption of leaf metabolism and subsequent leaf blackening.

Sucrose (2.5% to 5% w/v) significantly delayed or even prevented leaf blackening. The respiration rate of individual leaf and flower head on stems held in sucrose solution was 60% higher than those held in water. No ion leakage was found in leaves held in sucrose solution and soluble PPO activity remained low.

Leaf susceptibility to blackening differs between *P. neriifolia* R. Br. and *Leucospermum* 'Rachel', members from two different genera of the family Proteaceae. Leaf discs from *Leucospermum* did not turn brown in the dark, while *Protea* leaf discs browned within 48 hours in the dark. *Protea* had a higher dry weight percentage and protein content, as well as a greater total phenolic content. Polyphenol oxidase (PPO) activity was high in *Protea*, while none was detected in *Leucospermum* leaf tissue. A significantly higher pH was observed in *Protea* leaf homogenate. No significant difference was observed in ascorbic acid content in leaf

tissues of the two genera. However, a subsequent decrease in the ascorbic acid content was observed in *Protea* leaf extracts as they turned brown, then black. *Leucospermum* leaf extract remained green and appeared to slow the rate of extract browning when mixed with *Protea* leaf extract. An inhibitory effect on *Protea* PPO activity was found in *Leucospermum* leaf extract.

The present studies suggest that PPO activity plays an important role in *Protea neriifolia* postharvest leaf blackening. Depletion of leaf carbohydrates, or continued darkness, probably cause a loss of chloroplast membrane integrity and release and activation of membrane bound PPO. *Leucospermum* is not susceptible to the blackening due to lack of, or the presence of inhibitors of PPO activity.

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I. INTRODUCTION

Many *Protea* species have recently become ornamental cut flower crops in the international flower markets. In Hawaii, growing *Protea* as a cut flower started in 1960s at the Hawaii Agricultural Experimental Station (Parvin *et al.*, 1973). Commercial *Protea* growers exist now on the Islands of Hawaii and Maui. The Protea industry has grown into a multi-million-dollar enterprise with financial support from the state of Hawaii. Ninety percent of protea blooms originate on Maui (Kepler 1988). These Proteas are sold in the State of Hawaii, shipped to mainland U.S.A, and occasionally European countries. Other places where Proteas are grown for cut flowers include California U.S.A., Australia, New Zealand, as well as their native habitat--South Africa (Parvin *et al.*, 1973).

Protea, named by Linnaeus after the Greek mythological god, refers to any member of the family *Proteaceae*, which includes over 1,500 species of trees, shrubs, and herbs (Parvin *et al.*, 1973). However, *Protea* is also a genus name in the family. In the genus of *Protea*, *P. neriifolia* is one of the important cut flowers in Hawaii. The cut flower of *P. neriifolia* consists of a inflorescence called a flower head. A mature flower head is about 13 cm long and 6 to 8 cm in diameter. The inflorescence is classified as an involucrate capitulum (Rourke, 1980). On the involucral receptacle (5 to 6 cm in diameter), there are 300 to 400 individual flowers, called florets. The florets are surrounded by 3 to 5 layers of involucral bracts. The inner bracts are long, soft, and colorful, "tipped with dark 'fur' lending a tactile as

well as visual appeal" (Watson and Parvin, 1973). The outer involucral bracts are shorter, woody, and less colorful.

The flower is usually harvested when the inner bracts are beginning to unfold, and can last as long as 3 to 4 weeks in postharvest vase solution. The flower head is supported by a flower stem on which 20 to 30 green leaves are attached. The leaf is oval-shaped, about 10 cm in length and 3-4 cm in width. When green and fresh, the leaves add additional beauty to this cut flower.

The major problem of this cut flower is postharvest leaf blackening, that greatly reduces the flower's potential market value. After harvest, flower stems are usually packed in cardboard boxes of different sizes with or without a plastic liner. Without refrigeration, the leaves begin to turn black within 2 to 3 days from harvest. This greatly limits the shipping distance and time, and imposes a serious problem on Hawaiian growers since refrigerated transportation is expensive and in many cases impractical for Hawaii. Even when sold in the local market, freshly received flowers may rapidly lose their value due to leaf blackening if improperly handled; uncontrolled temperature and being held in the dark.

Postharvest leaf blackening has been observed world wide. Leaves of susceptible *Protea* species from California turn black within 3 to 7 days after harvest (McConchie *et al.*, 1991). In New Zealand, Bieleski *et al.* (1992) observed complete leaf blackening of *Protea eximia* within 8 days of harvest. In South Africa, Ferreira (1986) found that browning in leaves of *P. neriifolia* could be induced in 30 minutes, if the leaves were kept at high temperatures (60 and 65°C). The universality of

Protea leaf blackening was one of the justifications used for the approval of the formation of a Protea Working Group within the Section, Ornamental Plants, of the International Society for Horticultural Science in 1984. Active members of the Protea Working Group are from Australia, Israel, New Zealand, South Africa and U.S.A.

A. Previous Research

Researchers believe that the direct cause of the blackening is the oxidation of the abundant hydroxyphenols and tannins that occurs with loss of cell compartmentation (Paull *et al.*, 1980; Ferreira, 1983). However, the causes of the loss of cell compartmentation is unknown.

Protea has a relatively large flower head, removal of the flower head significantly delays the onset of the leaf blackening (Paull, 1980, Reid *et al.*, 1989). Some researchers suggest that competition for water between the flower head and the remaining leaves, creates water stress in the leaf cells (Paull, *et al.*, 1980) leading to a loss of compartmentation. Indeed, water loss from the flower head is high, varying from 25 to 50% of the water loss from a leafy stem with flower (Paull *et al.*, 1980). De Swardts *et al.* (1987) suggested that only 1% moisture loss may initiate blackening. Others have argued that it is competition for sugars between the flower head and leaves that leads to the depletion of leaf carbohydrates, and loss of cell compartmentation (Reid *et al.*, 1989). The flower head continues to grow after harvest with a high rate of respiration (McConchie *et al.*, 1991; Ferreira, 1986).

Many genera in the family of *Proteaceae* also produce nectar (Rourke, 1980). *P. neriifolia* is one of the species in the genus to produce the largest volume of nectar (Cowling and Mitchell, 1981). Fructose and glucose are the dominant carbohydrates. Removing flower head, girdling flower stem just below the flower head (Reid *et al.*, 1989), holding the flower stem in a bright light condition (Reid *et al.*, 1989), or adding appropriate concentration of sugar to the vase solution (Brink and de Swardt, 1986; Ferreira, 1986; Reid *et al.*, 1989) delays or even prevents leaf blackening. This strongly support the hypothesis that the leaf blackening may be due to depletion of carbohydrates in the flower stem. Bieleski *et al.* (1992) and McConchie *et al.* (1991) found that starch and sucrose concentration in leaves declined when flower stems were held in the dark but increased in the light, further supporting the idea that carbohydrate depletion could be the primary stress that initiates leaf blackening. However, the hypothesis of water stress leading to leaf blackening can not be totally ruled out.

B. Symptoms of *Protea* leaf blackening

The descriptions of the *Protea* leaf blackening disorder in literature are inconsistent. "Leaf browning" were used by some South Africa authors, others preferentially used the term "leaf blackening" (U.S.A. authors). The difference between these two terms probably lies on the degree of the disorder, or different perception by people. There has never been an explanation to this inconsistency. Nevertheless, four symptom types of this disorder has been described by de Swardt

(1987) in South Africa, based on the appearance of the leaf surface visible symptoms. The particular stress relating to each specific symptom of the leaf blackening disorder is unclear:

1) tip browning or blackening: the disorder initiated at the leaf tip;

2) marginal browning or blackening: the disorder initiated on the leaf margins and gradually spreads towards the midrib;

3) spot browning or blackening: the disorder initiated as individual spots on the leaf surface and enlarge progressively;

4) midrib browning or blackening: the disorder initiated in the immediate area of the midrib and spreads towards the leaf margins.

Although previous studies have suggested that carbohydrates drained by the flower head may cause depletion of the metabolites in the leaf, initiating leaf blackening, no direct evidence has been provided. The importance of water relation to leaf blackening has received less attention or been ignored; and the relationship to the blackening symptom with specific stress is not clear. The property of *Protea* leaf polyphenoloxidase has not been studied in detail.

In the present study, the symptoms of leaf blackening were characterized as to specific stresses. Direct evidence of carbohydrates movement from flower stem leaf to the flower head is provided by application of ^{14}C-sucrose to the leaf. The relationship between carbohydrate source (leaves) and sink (flower head) was studied by varying sink strength. The response to water stress and darkness leading to leaf blackening was also investigated. Some properties of the enzyme that

catalyzes the oxidation of the phenolics, the polyphenoloxidase (PPO) were determined. The relationship of this enzyme to leaf blackening was proposed.

II. LITERATURE REVIEW

A. Nature of Plant Tissue Blackening

Browning or blackening of plant tissue may be nonenzymatic, resulting from the Maillard reaction that involves a reactionable carbonyl compound and amines (Labuza and Schmidl, 1986; Nimiki, 1988) or from autooxidations frequently involving phenolic compounds (Cilliers and Singleton, 1989). However, most plant tissue browning or blackening are initiated by enzymatic oxidation of phenolic compounds. One of the enzymes catalyzing the oxidation of phenolic compounds is polyphenol oxidase (EC 1.10.3.1, PPO), also known as catechol oxidase, phenolase, and o-diphenol oxygen oxidoreductase. This enzyme catalyzes the oxidation of o-diphenols to o-diquinones as well as the o-hydroxylation of monophenols (Mayer and Harel, 1979; Butt, 1980). These initial products are very reactive quinones. These quinones can react with each other and other compounds, condense to form insoluble high molecular weight, deep brown or black pigments called Melanins (Labuza and Schmidl, 1986). The higher the molecular weight of the polymer formed, the darker the pigments.

PPO, a Cu-containing metalloenzyme, is present in substantial amounts in most plant tissue (Arnon, 1949). It exists as multiple forms in plant tissues (Lieberei *et al.*, 1981) with a molecule weight of 43 kilo Daltons for spinach (Lieberei *et al.*, 1981) and 45 kilo Daltons for broad bean (*Vicia faba*) leaves (Flurkey, 1985; 1986). The enzyme is nuclear encoded (Vaughn *et al.*, 1981, Vaughn and Duke, 1981b),

synthesized on cytoplasmic ribosomes and transported across the chloroplast membranes (Tolbert, 1973; Lax *et al.*, 1984; Vaughn and Duke, 1984).

In healthy green plants, PPO is localized exclusively in the chloroplast thylakoid membranes. The chloroplastic location of PPO has been confirmed by many investigators (Tolbert, 1973; Lax and Vaughn, 1991; Vaughn and Duke, 1981a; Vaughn and Duke, 1984; Sherman *et al.*, 1991). Cellular fractionation studies suggest that the pellets enriched in chlorophyll are also enriched in PPO (Arnon, 1949; Parish, 1972; Henry *et al.*, 1981). By coupling fractionation techniques with PPO cytochemistry, Henry *et al.* (1981) were able to confirm a plastid localization of PPO. Sherman *et al.* (1991), when conducting a survey of PPO distribution among 50 species ranging from aquatic green algae to terrestrial angiosperms by three different assay protocols, concluded that all species with PPO activity measured by spectrophotometric or gel assay had cytochemically-detected activity localized in thylakoid membranes. Treatment of mung bean plants with tentoxin, a cyclic tetrapeptide that specifically affects the plastids (Vaughn and Duke, 1981b), can result in the complete loss of PPO activity without affecting other organelles. Several higher plant plastid mutants lack PPO (DuBuy *et al.*, 1950). The loss of PPO in these chemically or genetically affected plastids strongly suggests that PPO is strictly a plastid enzyme in higher plants (Vaughn and Duke, 1984). Reports of PPO in other organelles were artifactural (Vaughn and Duke, 1984).

In some higher plants, PPO is bound to the membranes of chloroplasts and exist in a latent form (Tolbert, 1973; Mayer and Harel, 1979; Meyer and Biehl, 1981;

Sato and Hasegawa, 1976). *In vivo* PPO activity seems to be very low or non-existent in healthy tissue (Tolbert, 1973; Lieberei and Biehl, 1981). The latent form of PPO being activated upon release from the thylakoid membrane. Agents such as detergents that break down membrane structure activate PPO activity *in vitro* (Sato and Hasegawa, 1976). Trypsin (Tolbert, 1973) and fatty acids (Golbeck and Cammarata, 1981; Hutcheson and Buchanan, 1980) can also activate PPO. In spinach leaves, increases in PPO activity and its activation have been observed during senescence and ageing (Meyer and Biehl 1980, 1981). This increase is due to reduced binding of latent PPO to the thylakoid membranes and not to *de novo* synthesis, since the same amount of active PPO is present in isolated thylakoid membranes from senescent and non-senescent leaves after activation of the latent enzyme (Meyer and Biehl, 1981).

The vast majority of phenolic compounds such as tannins in higher plant cells are located in the vacuole (Baur and Walkinshaw, 1974). This compartmentation of substrates separated from the enzyme prevents *in vivo* oxidation from occurring. Once the plastid and vacuole contents are mixed, which usually happens during senescence or injury, browning occurs. Since PPO catalyses the oxidation with molecular oxygen of mono and dihydroxy phenolic compounds of the cell, regulation or suppression *in vivo* of this oxidase must occur (Tolbert, 1973). Upon crushing or aging of a cell, uncontrolled polyphenol oxidation results in browning (Tolbert, 1973; Meyer and Biehl, 1981). Browning or blackening during senescence is attributed to the increased PPO activity and the subsequently mixing with phenolic compounds,

as in the development of the pigmentation in black olives (Ben-Shalom *et al.*, 1977), and other dark brown or black, usually found in dead plant tissues. Extractable PPO activity has been found to increase and correlate well with fruit discoloration during postharvest storage of avocado (Kahn, 1975; Van Lelyveld and Bower, 1984; Van Lelyveld *et al.*, 1984; Bower and Van Lelyveld, 1985), banana (Jayaraman *et al.*, 1982), and mango (Joshi and Shiralkar, 1977; Venkaiah and Patwardhan, 1977). This activity increase is attributed to the release of membrane-bound pre-existing PPO and substrates into the cytosol, where browning occurs. Extractable PPO activity in spinach leaves during senescence increases 15 to 20 fold, due to the activation of previously synthesized enzyme (Meyer and Biehl, 1981). Mechanical injury during food processing causes mixing PPO with phenolic compounds thus browning, and is a major problem facing the food industry.

The role of PPO in higher plants is not clear. Some investigators have assumed that PPO is involved in phenolic compounds synthesis *in vivo* since PPO can readily convert monophenols to o-diphenols *in vitro* (Vaughn and Butt, 1969; Schill and Grisebach, 1973; Butt, 1980), but a number of argument have been proposed against this assumption (Vaughn and Duke, 1984). A role of PPO in photosynthesis rather than its phenol oxidase activity has been suggested since the chloroplasts thylakoid membranes are sites of intense photochemical activity (Vaughn and Duke, 1984) and PPO is located exclusively in the thylakoid membrane. Mayer and Harel (1979) proposed that PPO could provide an oxygen buffer. Vaughn and Duke (1981a) found that PPO existed only in those chloroplasts

that evolve high levels of oxygen. A reaction sequence illustrating the possible oxygen buffering of PPO has been proposed (Vaughn and Duke, 1984):

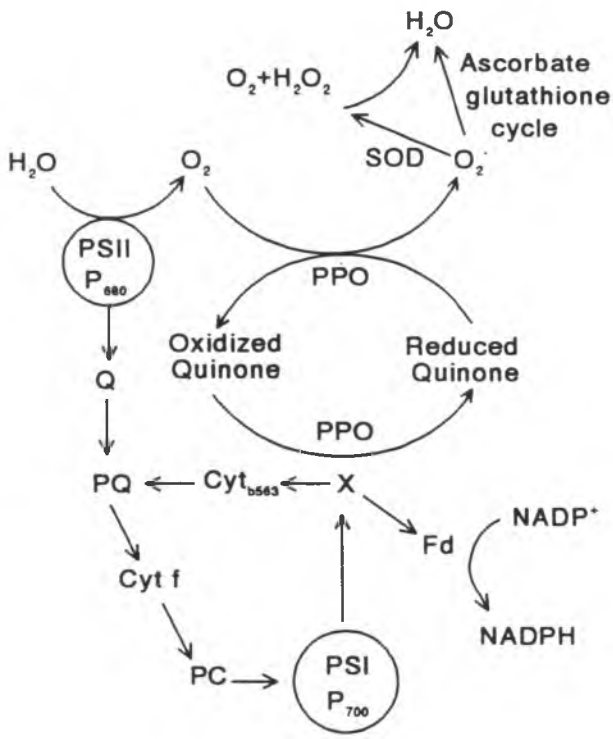
$$\text{Diphenol} + O_2 \xleftarrow[\text{PPO}]{} \text{diquinone} + H_2O \qquad (1)$$

Either ascorbate or NADPH could be used to regenerate the diphenol so that reactive diquinones are not accumulated (Vaughn and Duke, 1984). However, the Mehler reaction and the associated ascorbate-glutathione cycle can rid the chloroplast of excess oxygen (Halliwell, 1984; Loewus, 1988; Thomsen *et al.*, 1992), therefore this oxygen buffering function seems unlikely (Vaughn and Duke, 1984). The Mehler reaction produces superoxide which is then detoxified by superoxide dismutase (SOD) lowering the oxygen level (Halliwell, 1984). In photosynthetic cells the plastidic ascorbate-glutathione pathway is considered the major sequence involved in the elimination of active oxygen species (Thomsen *et al.*, 1992). Some functional relationship between photosystem II (PSII) and PPO has been proposed because there appears to be a strong correlation between the presence of PPO and the capacity for high levels of O_2 evolution in green tissues (Vaughn and Duke, 1984). Lax and Vaughn (1991), using sucrose density gradient fractionations of thylakoid membranes following detergent solubilization, found that PPO protein (by reactivity with anti-PPO antibody) and activity (based upon ability to oxidize dl-dihydroxyphenylalanine) were the only fractions enriched in PSII in leaves. Immunogold localization of PPO on thin sections revealed exclusive thylakoid labeling with a distribution pattern consistent with other PSII proteins, suggesting

that PPO is at least peripherally associated with the PSII complex. However, because no copper-containing proteins have been reported to be associated with water splitting, and bundle sheath cells of C_4 plants have PSII activity during early development stages (Vaughn and Duke, 1982) but no PPO activity (Vaughn and Duke, 1981a), the direct involvement of PPO and PSII is still unclear.

A role of PPO in pseudocyclic photophosphorylation suggested by Tolbert (1973) involves ATP production with O_2 as a terminal electron acceptor. Sherman *et al.* (1991) also suggest that the primary function of PPO is modulation of photosystem I reduction of molecular oxygen (Mehler reaction or pseudocyclic photophosphorylation). Under conditions of low oxygen tension, photosynthetic energy is normally directed to reduce intermediates necessary for biosynthetic processes while under high oxygen concentrations, energy is diverted to dissipate molecular oxygen via the Mehler reaction (Sherman *et al.*, 1991). A possible role of PPO in regulating pseudocyclic photophosphorylation by controlling the amount of Mehler reaction taking place has been proposed by Vaughn and Duke (1984). In this model (Model 1), quinones can mediate the Mehler reaction, and PPO could facilitate the reoxidation of quinones after reduction by X, allowing the quinone to act at catalytic levels. This function would have to be regulated (Tolbert, 1973). Growing evidence suggests that the Mehler reaction is well regulated in healthy chloroplasts (Robinson and Gibbs, 1982), however, the mechanism of regulation is unknown. Increased phenol oxidase function may be associated with loss of Mehler reaction function (Vaughn and Duke, 1984). Evidence that cells generate little or

no molecular oxygen such as bundle sheath cells of C_4 plants (Vaughn and Duke, 1981 a) and guard cells (Vaughn *et al.*, 1981) generally have no PPO support the theory that PPO is involved in the Mehler reaction.



Model 1. Possible role of PPO in regulating pseudocyclic photophosphorylation by controlling the amount of Mehler reaction taking place (Vaughn and Duke, 1984).

PPO may also play an important role in plant protection and disease resistance (Vaughn and Duke, 1984; Bell, 1981). Necrosis, or local lesions formed after pathogen invasion, is associated with race-specific resistance (Bell, 1981). This resistance is characterized by the formation of brown to black pigments (melanin) throughout the cell walls and the collapsed protoplasts. The intensity of the melanin

formation often is greatest in highly resistant plants, suggesting that melanins or their precursors contribute to resistance (Bell, 1981; Martyn *et al.*, 1979). Melanin formation, as discussed before, involves the oxidation of polyphenol compounds and PPO is one of the enzymes catalyzing the reaction. Upon injury or disease infection, the disruption of cellular compartmentation leads to the activation of latent PPO which reacts with phenolics released from the vacuole (Butt, 1980; Mayer and Harel, 1979). However, the increase in extractable PPO activity during senescence seems to have no obvious functional significance or survival advantage (Vaughn and Duke, 1984).

B. Postharvest Stresses and Cut Flower Senescence

Senescence has been described as a series of deteriorative processes that lead to diminishing metabolic activity and ultimately death of an organ or organism (Leopold, 1964; Ceppi *et al.*, 1987). Like many developmental process, senescence is under direct genetic control (Thomas and Stoddart, 1980). However, many environmental factors, such as light, temperature, water relations, mineral relations, and invasion by pathogens, as well as the correlative effects among the plant can influence senescence (Thomas and Stoddart, 1980; Nooden, 1988).

Flower senescence is generally rapid and predictable (Halevy and Mayak, 1979; Borochov, 1989). The flower is a complex organ composed of many different tissues, all of which senesce at different rates (Borochov, 1989). In many commercial cut flowers, it is usually the life span of the short-lived petals that determine the

effective life of the flower (Halevy and Mayak, 1979; Borochov, 1989). In *Protea*, however, the individual flowers are small and not attractive (Rourke, 1980). It is the bracts that give the cut flower brightness and commercial value. Therefore it is the life span of the bracts and the greenness of the flower stem leaves together determine the vase life of *Protea* cut flowers. There have been no known studies of flower bract senescence. Petal and leaf senescence studies of other commercial cut flowers should provide some background and direction in understanding *Protea* cut flower senescence. Biological stress, such as water stress and carbohydrate depletion (Thomas and Stoddart, 1980) can cause plant tissue senescence possibly leading to tissue browning or blackening, especially during the postharvest handling of most cut flowers.

1. Water relations

Water relations are very important in postharvest life of many cut flowers (Mayak, 1987). Rogers (1973) assigned the highest priority to the maintenance of flower turgidity being vital to the needs of the cut flower, as a high level of turgidity is necessary not only for development of flower buds to full-bloom maturity but also for the continuance of normal metabolic activity in the cut flower. The turgidity in flowers is dependent upon a balance between the rate of water supply and water loss (Rogers, 1973).

Water stress caused flower senescence has been reported for many commercial cut flowers (Halevy and Mayak, 1979; 1981; Mayak, 1987). Most cut

flowers are constantly under water stress because of an imbalance of water uptake and water loss when held in vase solution (Mayak 1987). This eventually results in wilting or shedding of flowers and termination of the vase life of most cut flowers (Halevy and Mayak, 1981). For example, *Dendrobium* flower begin to shed when the rate of water uptake fall below 1.0 g day^{-1} $spray^{-1}$ (Dai and Paull, 1991) and addition of a floral preservative to the vase water slows the decline in water loss and increases postharvest life. The reduced water uptake rate is caused by vascular blockage of the flower stem (Marousky, 1969; Halevy and Mayak, 1981). Several factors have been suggested to be involved in vascular blockage: microorganisms' growth in the solution and in the vascular system (Put and Jansen, 1989; Put, 1990; Larsen and Cromarty, 1967; Zagory and Reid, 1986), oxidation processes induced from harvesting injury (Durkin and Kuc, 1966; Burdett, 1970; Marousky, 1971), enzymatic plugging of the stem (Mayak *et al.*, 1974), and air embolism (Crafts, 1968; Durkin, 1980). The blockage of water transport system and subsequent reduced water uptake, causes water deficit in flower petal cells due to continuous transpiration from the cut flower (Marousky, 1969). When the rate of water loss from the tissue is higher than the rate of water uptake from the cut stem, then water stress results (Halevy and Mayak, 1981). Young, unlignified tissues lose their turgidity, and finally wilt, terminating the vase life of most cut flowers. A very well demonstrated phenomenon is the "bent-neck" in cut roses (Burdett, 1970). Applying germicides and chemicals (e.g. 8-HQC or 8-HQS,) that lower vase solution pH reduce microorganisms growth and improve water uptake in the cut flower stem,

thus prolonging cut flower longevity (Zagory and Reid, 1986; Marousky, 1969; 1971). Two reviews of cut flower water relations and senescence have appeared (Rogers, 1973; van Doorn, 1989).

Water stress not only changes the physical but also the biochemical status of the cell (Halevy and Mayak, 1979; 1981). For example, in cut Gerbera flowers, even when the water potential remained constant, the water content of petals and turgidity declined with time (Van Meeteren 1978, 1979a,b), indicating cellular physiology changes associated with senescence. To study short term effects of water stress, Mayak (1987) imposed a transient mild water stress and then returned the flowers to water. His data indicates increases microviscosity of membranes at the end of the stress period, and also increase in the hydrophobicity of the membrane, suggesting a compositional changes of membranes during water stress. These changes may be related to senescence. When the stress is released, the biophysical changes subside.

2. Carbohydrates depletion

Depletion of carbohydrates in cut flower has been considered as the second important aspect involving cut flower deterioration (Rogers, 1973). Depletion of stored materials through respiration is an important factor in cut flower deterioration, and the maintenance of a carbohydrate pool in the flower is important for promoting flower longevity (Sacalis and Durkin, 1972). During flower growth and development, the rate of respiration in many flowers rises to a maximum as the

flower start to open, followed by a gradual decline as the flower mature (Coorts, 1973). There is a second peak in the respiration drift that is considered to indicate the final senescence stage, and is assumed to be analogous to the climacteric rise in respiration of many fruits (Larsen and Frolich, 1969). The gradual decline in respiration with aging of flowers may be caused by a short supply of respirable substrates, mainly sugars. Treatments that delay the occurrence of the second peak also extended longevity of cut flowers. Coorts *et al.* (1965) found in roses, the preharvest respiratory rate is greater than the postharvest rate. Preservative-treated roses had a 40% higher respiratory rate than those in water, and sucrose is the major component of the preservative contributing to increased respiration, decreased transpiration, and delayed senescence. The keeping quality of roses is enhanced by preservatives that increases respiration, rather than those that inhibit it.

Along with carbohydrates, other macromolecules are also hydrolysed during flower senescence. Protein content is reduced, and protein degradation products such as smaller polypeptides and amino acids increase (Parups, 1971). A several-fold increase in ammonia levels was observed in senescing roses (Weinstein, 1957) and anthurium (Paull *et al.*, 1985). Amides such as asparagines and glutamines also accumulated. However, Woltz (1965) found that total free amino acids were depleted in the absence of adequate light in chrysanthemum leaves during storage. Weinstein (1957) proposed a model to explain the increase in free ammonia coinciding with the decline in respiratory activity and suggested a sequential order of these events. According to his model, depletion of free sugars initiates the onset

of hydrolytic processes of structural cell components including proteins to supply alternative substrates for respiration.

3. Ethylene effect

In addition to the problems of maintenance of turgor and prevention of depletion of respirable substrates, ethylene is another threat to cut flower longevity (Rogers, 1973). The senescence of flowers is often associated with increased production of ethylene (Borochov and Woodson, 1989), and many flowers are affected by exposing to small amount of ethylene gas. During the development of some cut flowers, especially carnations and roses, a climacteric rise in ethylene production signifies the progression of senescence (Mayak, 1987). A change in permeability of the tissue can be detected shortly thereafter.

C. Effects of Exogenously Applied Carbohydrates in Prolonging Cut Flowers Longevity

1. Carbohydrates provide cut flowers with respiratory substrates

Carbohydrate accumulation may occur during the growth of flowers on the plant, a preharvest criteria influencing postharvest vase life of cut flowers (Halevy and Mayak, 1979). Holley (1963) estimated that as much as 30% of a flower's potential keeping quality may be influenced by its preharvest environment with remaining influenced by postharvest handling. Among the factors the most important one seems to be total light energy (Halevy and Mayak, 1979), and the

main effect of the preharvest light conditions influencing longevity is by affecting the inherent carbohydrate levels (Rogers, 1962; Halevy and Mayak, 1979). Carnation (Mayak and Dilley, 1976) and chrysanthemum (Kofranek *et al.*, 1972) flowers produced during period of low light intensity age more rapidly than those produced during periods of high light intensity, and these differences can be reduced and even eliminated when the flower buds are treated with solutions including metabolic sugars (Halevy and Mayak, 1974b; Kofranek and Halevy, 1972; Mayak and Dilley, 1976). A relationship between potential keeping life and dry matter content of the cut flower at the time of harvest has been observed (Ketsa, 1989; Berkholst, 1989). Available food reserves in individual flower buds at time of cutting is regarded as the factor most affecting vase-life characteristics of *Dendrobium* 'Pompadour' cut flower, and the percentage of bud opening and vase life of opened buds was related to bud size; large the bud the longer the vase life (Ketsa, 1989). Berkholst (1989) found that high starch content in 'Sonia' rose corollas at picking may add quality to vase life of the cut flower. Since longevity of most cut flowers is correlated with carbohydrate level, flowers harvested late in the afternoon are supposed to last longer than those harvested in the morning (Rogers, 1962; Halevy and Mayak, 1979). This is true especially for flowers bearing leaves like roses (Halevy and Mayak, 1979).

Carbohydrate can also be supplied through the preservative solution. Metabolic sugars appear to be the only materials appreciably extending vase life (Marousky, 1969), therefore it is included in most preservatives for cut flowers. The

respirable substrate pool is composed mainly of sugars, and the size of the pool is affected by the rate of hydrolysis of starch and other polysaccharides (Ho and Nichols, 1977; Nichols, 1976b). Supplying cut flowers with exogenous sugar maintains the pool of dry matter and respirable substrates in flowers, thus providing for respiration (Coorts, 1973) and extending longevity (Coorts, 1973; Rogers, 1973). Several metabolic sugars such as glucose, fructose, and sucrose are active in this respect, while non-metabolic sugars like mannitol and mannose are not (Aarts, 1957; Halevy and Mayak, 1974b; Halevy and Mayak, 1981). Light given to some cut flowers such as chrysanthemums during holding and use periods improves keeping life of the product by extending the photosynthetic capacity of the foliage, thus providing carbohydrates to the flower (Woltz, 1965; Woltz and Waters, 1967).

An exogenous supply of sugars delays the onset of excessive protein degradation (Coorts, 1973; Parups and Chan, 1973) and also serves as a substrate for protein synthesis. The application of ^{14}C-sucrose to cut roses revealed that sucrose is incorporated into protein, ethanol soluble carbohydrate, starch, and ethanol insoluble material (Ho and Nichols, 1977; Sacalis and Chin, 1976).

2. Carbohydrates delay cut flower senescence by protecting membrane integrity

The plant cell plasma membrane consists of a lipid bilayer interspersed with proteins. It serves as cells outer boundary, separating the cytoplasm from the external environment. It allows the cell to take up and retain certain substances while excluding others. A disordered membrane will cause leakage of solutes from

the cell, and eventually leading to the death of the cell (Thompson, 1988; Borochove and Woodson, 1989).

The physical characteristics of membrane lipids are known to affect and regulate solute diffusion and uptake, membrane enzyme activity and hormone binding (Shinitzki, 1984). Changes in the composition and physical properties of the membranes have been shown to play a central role in the processes of senescence (Thompson, 1984; Borochov and Woodson, 1989). In a normal cell, the ratio of phospholipid (PL) to sterols (ST) is constant, and the proportion of unsaturated fatty acid is high. These keep the membrane in a fluid mobile state. In senescing cells, the first detectable change is a decrease in PL content and an increase in the ratio of ST to PL. The increase in this ratio leads in turn to a decrease in lipid fluidity (Borochov *et al.*, 1978; Borochov and Woodson, 1989). Increasing the proportion of saturated fatty acids to unsaturated fatty acids is also found in senescing cells, this also decreases the fluidity of the membrane. As membranes become less fluid, their protein components can no longer function normally (Borochov and Woodson, 1989). Membrane-bound enzyme activity decreases (Adam *et al.*, 1983) reducing the ability of the cell to exchange solutes with its environment. Aging leads to phase changes in the membrane from fluid to gel phase. The presence of lipids in a gel phase above a certain level is probably responsible for the increase in membrane permeability (Thompson, 1984; Borochov and Woodson, 1989). This leads to a loss of water from the cells, resulting in wilting. A loss of membrane integrity causes the leakage of toxic substances into the cytoplasm,

uncoupling electron transport chain and inability of mitochondria to produce ATP and ultimately cell death.

Sugars have a general protective effect on membrane integrity (Coorts, 1973; Parups and Chan, 1973). It has been found that when cut roses are held in a preservative solution containing sucrose, they show an improvement in flower quality and an increase in petal water content (Goszczynska *et al.*, 1990). The age-related decrease in membrane proteins, and senescence related reduction in membrane lipid fluidity of membranes isolated from sugar-treated flowers is delayed and the membranes have a higher phospholipid (PL) content than those from untreated flowers. Kaltaler and Steponkus (1976) isolated mitochondria from cut flowers pretreated with sucrose and found that respiratory values are maintained over a longer period of time than those not pretreated. They concluded that the main effect of applied sugar in extending longevity is to maintain mitochondrial structure and function. Sugar's ability to delay senescence of cut carnation flowers is related to its ability to support cell metabolism and to maintain membrane integrity has also been suggested by Acock and Nichols (1979).

Goszczynska *et al.* (1990) proposed two possible different mechanisms of the effects of sucrose on membrane lipid fluidity. One possibility is that the sugar molecules interact directly with the membranes. Hydrogen bonding and hydrophobic interaction have been suggested as possible mechanisms (Gaber *et al.*, 1986). Another possibility is that sugar might affect the properties of membrane lipids through interference with their metabolism. Goszczynska *et al.* (1990) found that

in water-treated flowers, the PL content decreases with senescence while in sucrose treated flowers the PL content per flower does not decline with age. Therefore they suggested that the PL level is the main factor controlling membrane integrity and water-holding capacity of rose flowers, and the effects of sugar on flower membrane integrity are exerted via modifications of lipid turnover.

3. Carbohydrates improve water balance in cut flowers

It is known that sucrose improves the water balance of cut flowers (Borochov *et al.*, 1976; Bravdo *et al.*, 1974; Halevy and Mayak, 1974b). Several possible mechanisms have been suggested. One suggested by Marousky (1971) is that the improved water balance in cut flowers is the effect of sugars on the closure of stomata and reduction of water loss through transpiration. He found that roses held in sucrose-containing solutions absorbed less water than roses held in water alone but sustained their fresh weight increases longer, this however cannot explain the great increase in water uptake found in other sucrose-treated flowers (Bravdo *et al.*, 1974; Halevy, 1976). The osmotic effect of the sugars on the flowers is another explanation. The translocated sugars accumulate in the flowers, increasing their osmotic concentration, and improve their ability to absorb water and maintain turgidity (Halevy, 1976; Halevy and Mayak, 1974b). Sugars maintaining membrane integrity and therfore preventing water leakage is another possibility.

4. Carbohydrates promote flower development during opening of cut flowers

The commercial development stage of the cut flower at harvest varies between different flowers. Many cut flowers, such as chrysanthemum, roses, gladioli or irises, and carnations, are harvested in the bud stage or before they are fully open. Flowers harvested at the earliest stages generally have good quality if they can fully open and develop in the vase (Halevy and Mayak, 1979). Harvesting flowers in the bud stage is preferable because they are easier to handle, less susceptible to detrimental environmental conditions like high temperature and ethylene (Barden and Hanan, 1972; Maxie *et al.*, 1973; Nichols, 1973) and have longer potential vase life. However, flowers harvested in the tight bud stage are incapable of developing to the commercial stage if placed in only water. Therefore, a solution containing nutrients necessary for opening and developing cut flowers must be provided. Such solution is termed 'opening solution' (Paulin and Jamain, 1982). Sugar is one of the main gradients of opening solutions. It provides a respiratory substrate and materials for growth. A deficiency of carbohydrates is found to be the main cause of the dying of freesia buds (Spikman, 1989) and application of sucrose in the solution benefitted the inflorescence development. Bud-cut carnations kept in opening solutions has about the same diameter, weight, and vase-life as those cut when mature (Paulin and Jamain, 1982). Chrysanthemum flower buds opened in solution have better quality and greater longevity than those opened on the plant in the greenhouse (Kofranek and Halevy, 1972). The effect of sucrose on opening of cut carnations and chrysanthemum flower buds is found to be better than that of glucose (Paulin and

Jamain, 1982; Kofranek and Halevy, 1972) and the optimum concentrations of sucrose for chrysanthemum flower bud opening vary with cultivars. However, for *Dendrobium* 'Youppadeewan' flower bud opening and vase life, glucose is found to be better than sucrose (Ketsa and Boonrote, 1990) and the optimum concentration is 4%. Germicide is usually included in the formula of preservatives and opening solutions to reduce the growth of bacteria and thus promotes the water uptake capability of the flower stem (Halevy and Mayak, 1981).

D. Factors Affecting Leaf Senescence

Leaf senescence differs from flower senescence in that it involves chloroplast senescence and does not always follow the same path as senescence of other cellular parts (Halevy and Mayak, 1979). A thorough review of leaf senescence has been provided by Thomas and Stoddart (1980). The most common and best-known symptom of green leaf senescence is yellowing, the result of chlorophyll degradation. Protein content declines during leaf senescence. Therefore, at the molecular level, chlorophyll and protein degradation have been used as indicators of leaf senescence. An early event in leaf senescence is the degeneration of chloroplasts functionally and structurally, and the loss of integrity of the chloroplast membrane system (Barton, 1966; Ikeda and Ueda, 1964; Shaw and Manocha, 1965; Batt and Woolhouse, 1975; Ben-David *et al.*, 1983). During leaf senescence, photosynthesis is gradually inactivated and the loss of activity is accompanied by a large decrease in the chlorophyll content of leaves (Sestak, 1977). Loss of photosynthesis is mainly

caused by loss of a functional unit of photosynthesis or by a decrease in the number of whole chloroplasts (Kura-Hotta *et al.*, 1987). The mechanism of leaf senescence is not well understood, and the processes are influenced by many factors (Thomas and Stoddart, 1980).

1. Water stress induced leaf senescence

Leaf senescence has been studied in some vegetables or economic crops, although the mechanism of senescence is not clear. Water status of a leaf strongly influence the progress of leaf senescence (Lipton, 1987). Enhanced water loss will enhance leaf senescence. Water stress has been found to modify chlorophyll and protein contents (De Silva, 1976), photosynthesis and respiration (Bunce, 1982; Hsiao, 1973) and increase membrane permeability (Mukherjee and Choudhuri, 1981). It is widely accepted that water stress leads to closure of stomata, thus increasing resistance for CO_2 entering the leaf, resulting in decrease of leaf internal CO_2 concentration and lowering of photosynthetic rate (Zrenner and Stitt, 1991).

2. Dark induced leaf senescence

Darkness has been widely employed to initiate leaf senescence in both detached and attached leaf studies (Goldthwaite and Laetscg, 1967; Thimann *et al.*, 1977; Wittenbach, 1977). This could be due to the fact that under dark conditions leaves cannot carry out photosynthesis. Light, in contrast to darkness, slows senescence (Thomas and Stoddart, 1980). The relationship between senescence

retardation and light intensity described by Goldthwaite and Laetscg (1967) for *Phaseolus* leaf discs, Thimann *et al.* (1977) for *Avena* leaf sections, suggests that light acts through its effects on photosynthesis rather than on photomorphogenesis. Light is not only essential for production of high energy compound (ATP) and the strong reducing power (NADPH) needed for CO_2 assimilation, but also essential for activating some enzymes necessary for carbon fixation (Buchanan, 1980). Decline in photosynthetic rate will reduce the availability of ATP and carbohydrate, either or both of which may exert a direct initiating effect on senescence (Thomas and Stoddart, 1980).

The leaf usually contains an excess of carbohydrates, mainly starch or sucrose (Coorts, 1973). These available carbohydrates provide the respirable material. One of the early and detailed work on respiration and senescence of leaves was conducted by Blackmam (1953) who first studied respiratory time drift on leaves of cherry-laurel (*Prunus laurocerasus*, Linn.) and of nasturtium (*Tropaeolum majus*, Linn.). Blackman divided the respiratory time drift into 6 phases. Phase 1, high level of respiration, the respirable material represented the available carbohydrates at time of harvest. Phase 2, the rapid fall of respiration that was considered common to almost all leaves. The decline was explained by, or associated with, a progressive exhaustive decrease of available carbohydrates, namely sucrose and the polysaccharides. Phase 3, low level of respiration, described as the period of a slow and steady carbon dioxide evolution. Phase 4, steady rise of respiration attributed to extensive protein breakdown, with the carbohydrates making a progressively

smaller contribution to the steady rise of carbon dioxide evolved. Phase 5, steady fall of respiration, associated with the decomposition of amides and amino acids. This phase is followed by a loss of cell integrity, which may be caused by a large amount of ammonia liberated. Phase 6, sharp rise of respiration found to coincide with the leakage of vacuole's contents into the intercellular spaces. Cut-flowers are not generally credited with the capacity for manufacturing food when lighted or of benefiting from illumination (Woltz, 1965). However, some cut flowers, for example, chrysanthemums, are affected by light during storage (Woltz and Waters, 1967). The keeping quality of chrysanthemum as well as some other cut flowers are determined by both flower and leaf appearance. Light is found to increase greatly the vase life of chrysanthemums by maintaining the carbohydrate food supplies in the leaves at useful level (Woltz, 1965). Chrysanthemum leaves on cut flowers are capable of carrying on significant amounts of photosynthesis during illuminated storage (Woltz, 1965; Woltz and Waters, 1967). Woltz (1965) found that light (intensity above 50 foot candles) preserved photosynthetic capacity, chlorophyll content, total free amino acids and the supply of metabolites in leaves of *Chrysanthemum morifolium* Ram. cut flowers, thus kept leaves green and functioning. The light effect diminished under conditions of relatively low light intensity (less than 50 foot candle). In cases where the yellowing of foliage is a problem, light treatment was postulated as beneficial (Woltz and Waters, 1967).

3. Source and sink competition for nutrients may cause leaf senescence

The end product of photosynthesis is a three carbon sugar, triose-phosphate (triose-P), that can be used to form starch granules, one of the major polysaccharides, inside the chloroplast. Triose-P can also be transported across the chloroplast membrane to the cytosol where sucrose, a disaccharide, is synthesized. There is a diurnal change in carbohydrate metabolism in green plant cells (Gordon *et al.*, 1980; Fondy and Geiger, 1982; Kalt-Torres and Huber, 1987; Kalt-Torres *et al.*, 1987; Rufty *et al.*, 1983; Servaites *et al.*, 1989; Hammond and Burton, 1983; Huber, 1989; Gerhardt *et al.*, 1987; Mullen and Koller, 1988). Under light, extra starch is synthesized, stored in that chloroplast as an insoluble granule. During the dark period, when there is a demand for soluble carbohydrates for respiration and growth, starch is degradated, and the degradated products are used to form sucrose, that can be used up immediately, stored or transported to other organs through the phloem. A thorough review of sugar metabolism and compartmentation has been recently provided by Hawker *et al.* (1991).

Source organs are the organs that export assimilates to other organs and sink organs are the organs that import assimilates from source organs (Ho *et al.*, 1987). The dominant primary source of assimilate are leaves (Gifford and Evans, 1981), although green stems and floral organs (Kozlowski, 1992) can sometimes make substantial contributions. Mature leaves are the place where extra sugars are formed during photosynthesis and transported to other organs where sugars are needed for metabolism or storage (Hawker *et al.*, 1991). Therefore photosynthetic leaves are a

typical "source" organ. During photosynthesis sugars formed in the mesophyll cells are transported through phloem to sink organs, mainly in the form of sucrose. A reproductive organ, such as flower, fruit, or seed, is a typical sink organ. The ability of a sink organ to import assimilate is called "sink strength" (Ho, 1988) and is determined by sink size and activity (Daie, 1985):

$$\text{Sink strength (g/day)} = \text{Sink size (g)} \times \text{Sink activity (g/g/day)} \qquad (2)$$

Transportation of sugars from a source, the leaves, to sinks, the corollas has been found in some cut flowers. Sacalis and Durkin (1972) studied ^{14}C movement in cut roses and carnations using a pulse of ^{14}C-sucrose and a distilled water chase, and they found that the label moved selectively to leaves and stems during the pulse. During the chase, however, ^{14}C moved from leaves and stems into the flower heads. By girdling cut rose stems, they were able to show that assimilates are translocated from leaves and stems to flower heads via the phloem.

Leaf senescence induced by growth and development of reproductive organs has been studied in many monocarpic crops (Leopold, 1961; Crafts-Brandner and Egli, 1987; Wittenbach, 1982; Nooden, 1984; Purohit, 1982; Ho and Below, 1989; Ho *et al.*, 1987). This monocarpic leaf senescence is attributed to the nutrient drain by the reproductive organs since removal of reproductive organs has been shown to prevent leaf senescence. For example, *Helianthus annuus* L., a monocarpic plant, undergoes a rapid senescence as its fruits mature and this senescence can be prevented by surgical removal of the head at an early stage (Purohit, 1982). The removal of the head from the plant or fruits from the head at initial stages of fruit

growth and development prevented chlorophyll loss to a certain extent in *H. annuus* L. leaf (Purohit, 1982). Wittenbach (1982, 1983) found that leaves of depodded soybean plants accumulated more dry weight, and retained chlorophyll and protein longer than leaves of podded plants. He also found that depodding resulted *de novo* synthesis of four polypeptides and accumulation of starch. Therefore, he concluded that depodding altered leaf function (became a sink) rather than delaying or preventing senescence. Crafts-Brandner *et al* (1984) found that in soybean plants, pod removal affected partitioning of plant constituents, with leaves and stems of depodded plants serving as a major alternate sink for accumulation of dry matter, reduced N, phosphorus, and nonstructural carbohydrates (primarily starch). Ho *et al* (1987) also noticed that leaves of headless sunflower plants retained or accumulated more N, P, soluble protein, and dry weight than leaves of plants with heads and head removal delayed the rate of development of leaf senescence for the greenhouse-grown plants. Crafts-Brandner and Egli (1987) found that deflowering in soybean plants delay leaf abscission and a slower rate of chlorophyll loss.

Another theory for monocarpic leaf senescence induced by reproductive organ growth concerns the hormones or hormonal balance as a potential trigger of senescence. This theory has been proposed by Leopold and Kriedemann (1975), a senescence-inducing compound is synthesized in the seed of soybean plants and transported via the xylem to leaf. However, such compound has never been isolated.

E. Summary

Enzymatic browning or blackening in plant tissue is caused by oxidation of phenolic compounds presented in plant cells. This oxidation is catalyzed by polyphenol oxidase (PPO) which in healthy leaf tissue is located in chloroplast membrane in a latent form. Upon injury or senescence, PPO is released from the membrane and become activated. When mixed with phenolic compounds leaked from vacuole, oxidation takes place. Quinones formed from the oxidation react with each other or with proteins, amino acids, to form deep brown or black polymers, thus give the tissue a brown or black appearance.

Water stress, or carbohydrates depletion developed during postharvest handling can influence cut flower senescence, hence the leaf blackening process. Adverse environmental conditions such as darkness, or growth of reproductive organs may cause the depletion of carbohydrates in leaves, thus inducing leaf senescence. In PPO containing tissue, this senescence may lead to release and subsequently activation of PPO from chloroplast thylakoid membranes, oxidation of phenolics, and eventually the browning or blackening of the leaves.

III. HYPOTHESES AND OBJECTIVES

The general hypothesis is that *Protea* leaf blackening disorder is due to water or carbohydrate stress.

Specific hypotheses arising from this general hypothesis are:

1. Water stress, a leading factor causing most commercial cut flower senescence, may cause *Protea* leaf blackening;

2. Darkness induces leaf senescence, releases and activates polyphenol oxidase from the break down of the chloroplast membrane. Therefore darkness can induce *Protea* leaf blackening. The effects of light and sucrose are to provide leaves with respiratory substrates and to protect chloroplast membrane integrity, thus preventing leaf senescence and leaf blackening.

3. Flower head is a major sink for carbohydrates. Its growth and nectar production can significantly affect leaf carbohydrate status on the cut flower stem. Depletion of carbohydrates in leaves leads to leaf blackening. Providing flower stems with sugars or bright light and removal of flower head effect may delay or prevent leaf blackening.

4. Polyphenol oxidase (PPO) activity plays an important role in causing tissue blackening. Species or genera that do not show leaf blackening may lack PPO activity or have inhibitors of PPO activity.

The specific objectives of this study were:

1. To determine whether water stress causes leaf blackening, and if so, what the symptoms are;

2. To determine whether darkness induces leaf blackening, and if so, what the symptoms are;

3. To determine the relationship between sink (flower head) demand and source (leaf) supply, and the role of flower head on leaf blackening;

4. To measure leaf tissue polyphenol oxidase (PPO) activity, substrate availability, reducing agent content, inhibitory effect of PPO activity, and relate them to leaf blackening.

The above objectives were addressed by the following studies.

1. Water stress and leaf blackening: individual leaves were subjected to a rapid water stress, and the relationship between leaf fresh weight loss and leaf blackening determined;

2. Darkness-induced leaf blackening: Leaf discs and individual leaves were used to compare leaf blackening under light and dark conditions;

3. Effect of flower head on leaf blackening: The characteristics of flower head growth, respiration, and nectar production after harvest was correlated with leaf blackening. Direct evidence of sucrose transportation from source (the leaves) to sink (flower head) was provided by applying radioactive sucrose to a source leaf; The relationship between leaf blackening and flower senescence and number of leaves on a flower stem was determined;

4. PPO activity during the development of leaf blackening was determined. PPO activity, substrate availability, reducing agent content, inhibitory effect of PPO

activity, were measured, and compared with another genus that does not show leaf blackening.

IV. GENERAL MATERIALS AND METHODS

A. Plant Materials

Protea neriifolia 'Pink Mink' was used in most of the studies. Cut flowers were provided by the University of Hawaii Experimental Station, at Kula, Maui. Cut flower stems ca. 45 cm long (including a 10 to 13 cm long flower head), with ca. 25 leaves on the flower stem and, for most studies, a flower head at commercial harvesting stage (the inner bracts just begin to unfold) were used. For studying flower head growth after harvest, flower heads at different growth stages were harvested. The flowering season for *P. neriifolia* is from September to December, therefore most experiments were done during this period from 1988 through 1991.

Flower stems were harvested in the morning, packed in a cardboard box without plastic cover, and air shipped to Honolulu and arrived in our laboratory on the same day. After arrival, the cut flowers were sorted, basal stem recut (at least 2 cm) and placed into a flask containing 1 liter of deionized water or other chemical solutions prepared in deionized water. Flowers were then placed in an evaluation room and provided with white inflorescence light (PAR of 45 μmol m^{-2} s^{-1}) in 12 hr light/12 hr dark cycle and maintained at a temperature of 20 to 22°C. An evaporator was installed to provide 60% to 80% relative humidity in the room.

B. Vase Life Evaluation

Flower head and leaf conditions for each stem were evaluated every other

day. The flower head condition was evaluated as scales according the degree of wilting and discoloration of the inner and outer bracts respectively:

Scales: 0. Field condition;

1. Slightly discoloration;

2. Slightly wilting;

3. Moderate wilting;

4. Severe wilting.

The rate of leaf blackening was evaluated based upon the relative area of leaves with darkened surface as a percentage of total leaf surface area:

Scales: 0. No blackening;

1. 1% to 10% leaf area darkened;

2. 10% to 30% leaf area darkened;

3. 30% to 60% leaf area darkened;

4. 60% to 90% leaf area darkened;

5. 90% to 100% leaf area darkened;

The time from harvest to 50% leaf blackening (i.e. scale 3) was used to calculate vase life.

C. Statistical Analyses

At least five stems were used for each treatment and all experiments were repeated at least twice. Treatment results were cross-checked with similar treatments in other experiments. Mean comparisons were made using analysis of variance and

the Duncan-Waller multiple range test (SAS Institute, Inc., Cary, N.C.), and regression analysis was done by using the General Linear Model (GLM) procedure (SAS Institute, Inc., Cary, N.C.).

V. WATER STRESS AND LEAF BLACKENING

A. Introduction

The hypothesis of this study is that water stress, a leading factor causing most commercial cut flower senescence, can cause *Protea* leaf blackening. Therefore, the objective of this study is to determine whether water stress causes leaf blackening, and if so, what the symptoms are.

This chapter deals with the relationship between leaf and stem water status and leaf blackening. The type and development of leaf blackening symptoms under water stress were compared to published reports and observations.

B. Materials and Methods

1. Individual leaf study

Water loss determination. Rapid water stress was induced using individual mature leaves. Five individual mature green leaves (ca. 30 cm^2) were detached from the center of 5 flower stems upon arrival from the field. The leaves were placed on a bench without water in the evaluation room (PAR 45 $\mu mol\ m^{-2}\ s^{-1}$ 12 h light/dark, 20°C, RH 60 to 80%). Fresh weights were measured every day and weight loss was used to estimate the water loss.

Leaf blackening under dark condition. Five mature green leaves were detached from 5 flower stems upon arrival. They were inserted into 5, 50 ml glass tubes each containing 5 ml deionized water and then placed into completely darkness condition.

Symptom evaluation. Blackness and the symptoms of dark tissue development of the leaves subjected to water stress and darkness were evaluated subjectively by visual means. After the leaves had become completely darkened, their degree of blackness were determined objectively with a Minolta Chroma Meter CR-100 (Minolta Corporation, Ramsey, New Jersey, U.S.A.) having an 8 mm^2 measuring area. The Minolta Chroma Meter CR-100 is a color analyzer for measuring reflected subject color. It utilizes high-sensitivity silicon photocells filtered to match CIE (Commission Internationale de I'Eclairage) Standard Observer response. Chromaticity can be measured in L*a*b* (CIE 1976). CIE "a": +red/-green; "b": +yellow/-blue; and "l": +light/-dark.

2. Flower stem study

Preservative treatment. The water status of leaves attached to flower stems held in water or Florever solution (20 g l^{-1}) during the vase period was examined every 4 days over 16 days. Florever is a cut flower preservative manufactured by Smithers-Oasis (Smithers-Oasis, Kent, Ohio / Bakersfield, California, U.S.A.), which contains sugar, and anti-microorganism agents. Relative water content (Kramer, 1983) was used to express the water status in the leaf rather than fresh weight loss used for individual leaf study. Twenty leaves were taken from 5 different flower stems during each measurement, and 5 leaf discs (1 cm in diameter, main vein excluded) were taken from one of these leaves and weighed (fresh weight), placed in water overnight and weighed again (turgid weight). They were oven dried (80°C

for 48 hr) and weighed (dry weight). The relative water content (RWC) was calculated as follows:

$$RWC = \frac{\text{fresh weight - dry weight}}{\text{turgid weight - dry weight}}$$

This ratio represents the water content in the leaf at the time when measurement was taken. Five flower stems were used for each treatment. The darkening of the leaves was evaluated as described in the General Materials and Methods section and was compared to the RWC of the leaf during the vase life period.

Sucrose treatment. Five flower stems with foliage were held in deionized water or in 5% (w/v) sucrose in the evaluation room after arrival from the field. Water was added to the vase solution during the experiment as needed to maintain the volume. Fresh weight loss from the flower stem, flower senescence and leaf blackening were recorded every other day.

Measurement of stomata apparatus. Leaf stomata aperture of leaves still attached to flower stem was measured with a LI-6000 Portable Photosynthesis System (Li-Cor, Inc., Nebraska, U.S.A). At least 3 measurements were taken every other day at 3 pm. The results were expressed as stomatal resistance (s. cm^{-1}).

C. Results

1. Individual leaf study

Individual leaf fresh weight decreased to 85% of original weight within one

day after being placed on the bench without water (Figure 1.1). Yellowing of leaves started on day 2 when fresh weight had dropped to 78% of original weight. On day 3, when fresh weight had dropped to about 70%, browning began to appear on the leaf surface. The brownness first developed at the midrib area and spread outward. There was a rapid water loss between day 3 and day 4 (about 20%) and water loss reached 45% by day 4. Rate of browning increased during this period, by day 4, 90% of the leaf area had become brown. Water loss rate then declined to about 5% per day when the leaf was 100% brown and the fresh weight below 50% of original weight. Leaves with 100% browning had positive "a" value of 1.55 comparing to -6.15 for a fresh green leaf (Table 1). There was no significant difference in "b" value and in lightness ("l") between fresh green and water stressed brown leaves (Table 1.1).

Blackening on detached leaves held in deionized water in the dark started from the edge of the leaf and spread inward. There was no yellowing or browning before the blackening occurred. The "a" value on the colorimeter was +3.57, significantly higher than for green leaves and leaves under water stress. The "b" and "l" values of the darkened leaves were significantly lower than the fresh and water-stressed leaves (Table 1.1).

2. Flower stem study

Leaf water loss on the flower stem. Relative water content (RWC) of leaves on flower stems held without preservatives declined rapidly. RWC started to decline 4 days after harvest (Figure 1.2), from more than 90% of RWC to about 50% of

Figure 1.1. Fresh weight loss of individual leaf without water as expressed as percentage of original fresh weight (+------+) after harvest. The browning of the leaf (o------o) was determined visually. Bar = $\pm$SE, n = 5.

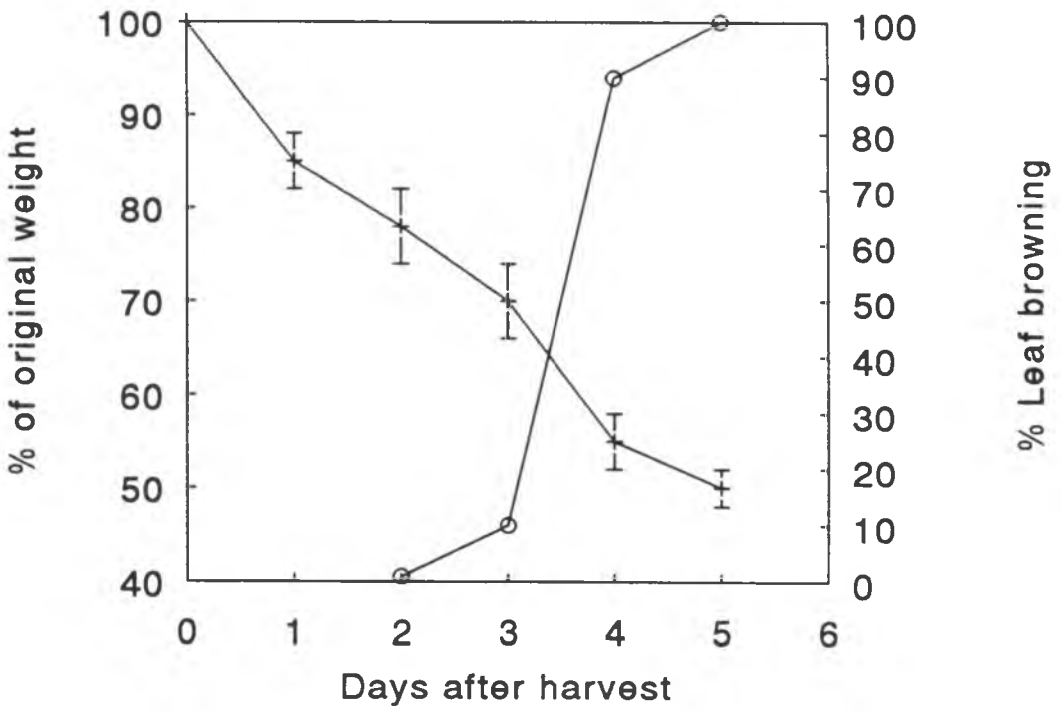
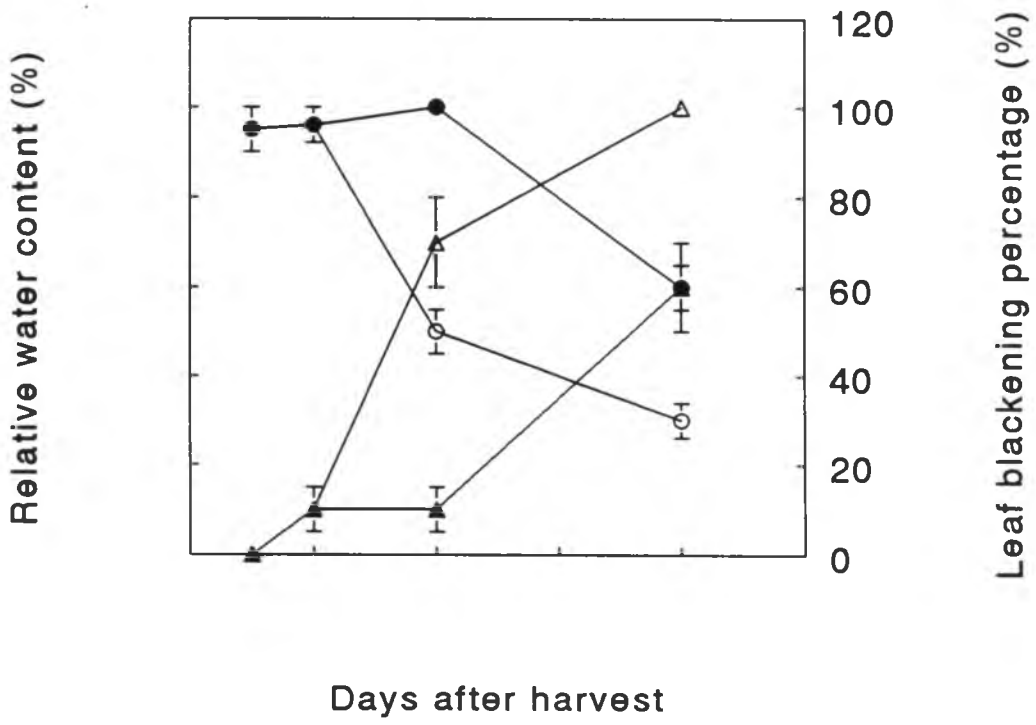

% of original weight
100
90
80
70
60
50
40
0
1
2
3
4
5
6
Days after harvest
100
90
80
70
60
50
40
30
20
10
0
% Leaf browning

Table 1.1. CIE "a" value (+ red/-green), "b" value (+ yellow/-blue), and "l" value (+ light/-dark) of detached and water stressed leaves held in a lighted room (PAR 45 μmol m^{-2} s^{-1} 12 h light/dark, 20°C, RH 60 to 80%). Fresh leaves were used as the control. CIE "a", "b", and "l" values of detached leaves held in deionized water in the dark were also determined.

Leaf treatment	CIE "a" value[z]	CIE "b" value	CIE "l" value
Control	-6.15 c	+10.00 b	+42.20 b
Water-stressed	+1.55 b	+11.48 b	+42.26 b
Leaves held in the dark	+3.57 a	+3.27 a	+33.24 a

[z]Data were analyzed by Duncan-Waller multiple range test, means followed by the same letter were not significantly different ($P<0.05$, $n = 5$).

Figure 1.2. Relative leaf water content and leaf blackening changes after harvest, o----o relative water content of flower held in deionized water, ●------● relative water content of flower held in "Florever" (20 g l^{-1}), ▵------▵ leaf blackening of flowers held in water, and ▲-----▲ leaf blackening of flowers held in Florever (20 g l^{-1}). Bar = MSE, n = 5.


Relative water content (%)
120
100
80
60
40
20
0
Leaf blackening percentage (%)
Days after harvest

RWC, 8 days after harvest. Leaf blackening (measured as dark area on the flower stem) was found to increase greatly, from 10% to 70% blackening during this 4-day period. RWC continued to decline gradually with gradually increasing leaf blackening. Sixteen days after harvest, RWC in leaf was ca. 20% when leaf blackening reached 100%.

Preservative solution prevented any decline in leaf RWC for 8 days after harvest and delayed the onset of leaf blackening for four days compared with water only (Figure 1.2). The RWC in the preservative treated leaves remained high (more than 90%) until 8 days after harvest and then declined gradually to about 50%, 16 days after harvest. Leaf blackening increased gradually, starting 8 days after harvest. The leaves of the preservative treated flower stem did not reach 100% blackening.

Effects of sucrose. Fresh weight loss from the cut flower stem held in water started within 2 days after harvest (Figure 1.3). The rate of fresh weight loss increased dramatically after day 2. By day 8 after harvest, the flower stems were 55% of original fresh weight.

Leaves of flower stems held in water started to blacken 2 days after harvest (Figure 1.3). Blackening increased rapidly so that four days after harvest, 50% of the leaves had blackened. Eight days after harvest, 100% of the leaves were fully blackened.

Flower stems held in 5% sucrose solution initially gained almost 5% in fresh weight (Figure 1.3). The highest fresh weight gain (about 5%) occurred 4 days after harvest, then declined. Eight days after harvest, the fresh weight of the flower stem

Figure 1.3. Fresh weight and leaf blackening changes after harvest. Percentage of original fresh weight of flower held in water (o-----o, Y_1), and in 5% (w/v) sucrose solution (●------●, Y_2). Percentage of leaf blackening on flower stems held in water (▵------▵, Y_3), and in 5% (w/v) sucrose solution (▲------▲, Y_4). Data points are means of 20 leaves. Regression analysis:

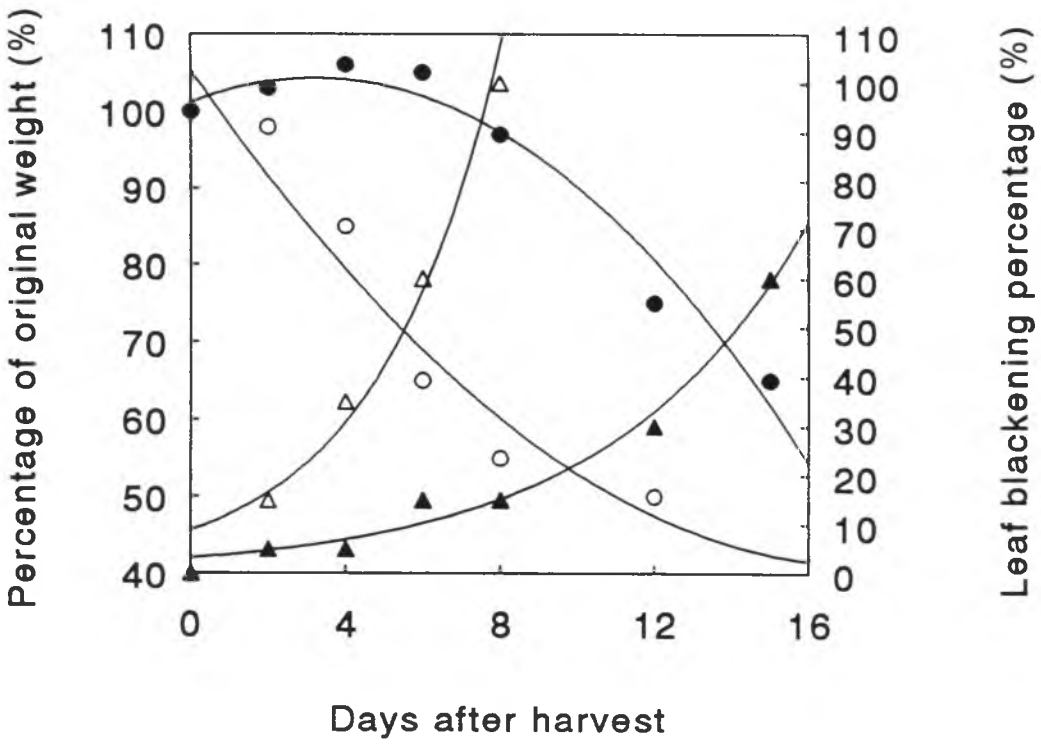
$Y_1 = 105 - 7.32X + 0.21X^2$, $r^2=0.97^{***}$;

$Y_2 = 101.15 + 1.97X - 0.31X^2$, $r^2=0.98^{***}$;

$Y_3 = 8.87 + 0.31X^2$, $r^2=0.99^{***}$;

$Y_4 = 3.21 + 0.19X^2$, $r^2 = 0.97^{***}$.

where X = days after harvest. *** significant at 0.1% level, n = 20.


Percentage of original weight (%)
110
100
90
80
70
60
50
40
0
4
8
12
16
Days after harvest
110
100
90
80
70
60
50
40
30
20
10
0
Leaf blackening percentage (%)

was almost the same as the original fresh weight. Then weight loss became rapid, 15 days after harvest, 25% of the fresh weight was lost. There was a quadratic relationship between weight loss and days after harvest for flower stems held in 5% (w/v) sucrose (Figure 1.3).

The relationship between percentage of leaf blackening and days after harvest in sucrose treatment was also exponential, but the rate of leaf blackening was slower when compared to the water treatment (Figure 1.3). There was a 4 day lag period before leaf blackening commenced, and then blackening increased at a slower rate than when held in water. Twelve days after harvest, only 50% of the leaves had blackened. The leaves on the flower stems treated with sucrose never reached 100% blackening during the 15 days experiment time.

Stomata aperture. Flower stems held in water had lower stomata resistance (Figure 1.4). However, the resistance increased as holding time increased. Sucrose treated flower stems had significantly higher stomata resistance (Figure 1.4). The resistance increased for 8 days after the start of treatment. The high level of resistance remained steady for another 2 days then declined. The lowest level of resistance was observed 14 days after harvest.

D. Discussion

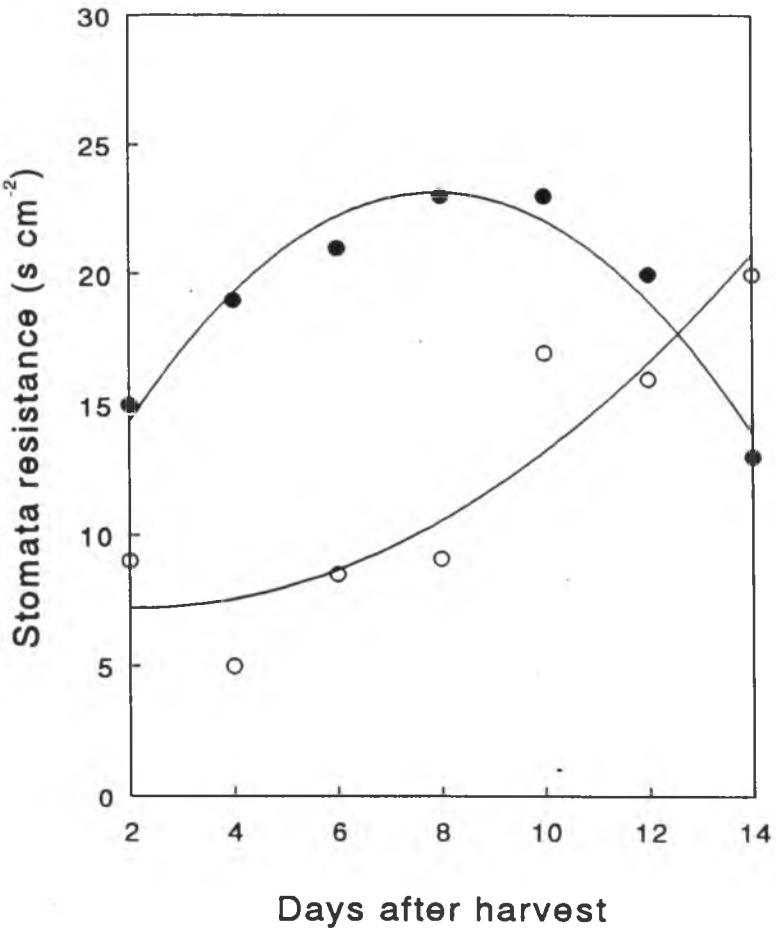
Postharvest water relations of cut flower vase life are considered to be the major factors determining flower vase life (Rogers, 1973; Coorts, 1973; Halevy and Mayak 1979, 1981). Leaf water loss did induce *Protea* leaf browning which began

Figure 1.4. Stomata apparatus changes after harvest. Stomata resistance of leaves on flower stems held in water (o------o, Y_1), and in 5% (w/v) sucrose solution (●------●, Y_2). Data points are means of 3 measurements. The regression analysis:

$$Y_1 = 9.04 - 2.03X + 0.85X^2, \qquad r^2 = 0.88^{**};$$

$$Y_2 = 7.43 + 7.93X - 1X^2, \qquad r^2 = 0.97^{***}.$$

where X = days after harvest. **, *** significant at 1%, 0.1% respectively.


Stomata resistance (s cm^{-2})
30
25
20
15
10
5
0
2
4
6
8
10
12
14
Days after harvest

after 2 days of detachment and without water, with the leaves becoming completely brown within 4 days when the fresh weight of leaves declined to less than 50% of the original fresh weight (Figure 1.1). However, the symptoms of detached leaves browning developed during rapidly induced water stress were different from symptoms found on detached leaves held in deionized water in the dark. This browning had a higher "a" value than green leaves, but lower than leaves blackened in the dark (Table 1.1). The "b" and "l" values of brown leaves were not significantly different from the green leaves but significantly higher than blackened leaves (Table 1.1), indicating that leaves under water stress were less black than leaves blackened in the dark. The browning in rapidly induced water stressed detached leaves started first from the leaf midrib and then spread outward, coincident with the so called "midrib browning" described by de Swardt (1987). The symptom of leaf blackening developed in the dark resembles the "marginal blackening" described by de Swardt (1987).

Water loss, expressed as loss in fresh weight, from flower stems held in water started 2 to 4 days after harvest (Figure 1.3), and coincides with the onset of leaf blackening. As flower stems continued to lose water rapidly the rate of leaf blackening increased, 50% of leaves had become black when about 30% of water was lost and complete black when 45% fresh weight were lost (Figure 1.3).

Leaves still attached to the flower stem held in water started blackening 2 to 4 days after harvest (Figure 1.2 and 1.3) and were completely black 8 days after harvest (Figure 1.3). Similar observations have been made with the same species

(McConchie *et al.*, 1991) and with another species, *P. exima* (Bieleski *et al.*, 1992). The onset of the leaf blackening and the complete leaf blackening can be delayed by continuously holding the flower stem in sucrose or commercial preservatives containing sugars (Figure 1.2 and 3). Sucrose has been found to induce stomata closure, retain water content in the cut flower petals, thus prolong cut flowers longevity (Halevy and Mayak, 1979; 1981). Leaves on flower stem held in sucrose (5% w/v) had stomata resistance twice that of the control (water only) (Figure 1.4), retain higher flower fresh weight for a longer period of time (almost 50% higher at 8 days after harvest), and delayed the onset of leaf blackening to 8 days after harvest (Figure 1.3). Preservative vase solution gave similar results, with the delay in decline of leaf relative water content, and the onset of leaf blackening to 8 days after harvest (Figure 1.2). Both sucrose and preservative prevented complete blackening for up to 16 days on attached leaves.

Sucrose and preservative delayed the onset of the leaf blackening, but did not prevent it. The onset of blackening started 8 days after harvest. The reason for this was unknown. Perhaps when water was added to the vase solution to keep the vase volume, the solution was diluted. The effects of sucrose or preservative might be diminished some days after harvest, and leaf blackening began.

This study confirms the hypothesis that water stress causes *Protea* leaf discoloration, but the degree and the symptoms are different from those induced by darkness. Whether these differences are serious enough to make a case for different mechanisms is unknown. Therefore further study is needed.

VI. DARKNESS INDUCED LEAF BLACKENING

A. Introduction

Darkness induces leaf senescence (Goldthwaite and Laetsch, 1967; Thimann *et al.*, 1977; Wittenbach, 1977). During leaf senescence, polyphenol oxidase (PPO) is released and activated from the break down of the chloroplast membrane (Meyer and Biehl, 1980, 1981; Vaughn and Duke, 1984). PPO catalyses the oxidation of phenolic compounds, a reaction required for forming brown or black pigments (Vaughn and Duke, 1984). Therefore the hypothesis of this chapter is that darkness induces *Protea* leaf senescence, releases and activates PPO from the chloroplast membrane. The effects of light and sucrose are to provide leaves with respiratory substrates and to protect chloroplast membrane integrity, thus preventing leaf senescence and leaf blackening.

The objectives of this chapter are to determine the effect of darkness on *Protea* leaf blackening, and the role of light and sugar in preventing leaf blackening.

B. Materials and Methods

1. Leaf disc studies

Light (12 h) and dark incubation. Leaves were detached from flower stems after arrival from the field and washed with deionized water. Leaf discs (1 cm in diameter) were cut while standing in 10 mM ascorbic acid solution (to prevent phenolics oxidation), and rinsed with deionized water. Ten leaf discs were floated in a 125 ml flask containing 30 ml water, with 3 replicates per treatment. The leaf

discs were subjected to 12 hr light/dark cycle (45 μmole s^{-1} m^{-2}) or in complete darkness by wrapping the flasks with aluminum foil. Discs were incubated for 72 hours at 20 to 22°C. In some experiments, 5 mM $CaCl_2$ was used and compared to water alone. Sucrose (2.5%), ascorbic acid (10 mM), diphenylamine (DPA, 10 mM), dithiothreitol (DTT, 10 mM), and sodium bisulfite (SBS, 10 mM) were also tested in complete darkness. Browning or blackening of the leaf discs was determined every 24 hr. Browning of the bathing solutions was determined spectrophotometrically by measuring absorbance at 490 nm every 12 hr.

Chlorophyll fluorescence determination. Chlorophyll fluorescence of each leaf disc was measured with a Plant Productivity Fluorometer (Model SF-20, Richard Brancker Reserch LDT., Ottawa, Canada) 72 hours after the start of incubation and compared with leaves that had just arrived from the field. Leaf or leaf discs were covered with a black cloth for 10 to 30 min before fluorescence was determined. A brief red light (5 seconds) was applied through the probe, and the initial, peak, and terminal phases of the chlorophyll fluorescence evolution were recorded. The initial value was designated as original fluorescence (Fo), and the peak value as maximum fluorescence (Fm). Fm minus Fo was designated as Fv, and the ratio of Fv/Fm obtained from leaf discs of above treatments were compared with those from fresh leaves. The Fv/Fm of a fresh leaf was used as a measure of green cell viability.

Chlorophyll, protein, and ascorbic acid content. Chlorophyll and protein content of the leaf discs were determined 72 hr after the start of incubation and

compared with a fresh leaf. Three leaf discs were homogenized with 5 ml 80% acetone, and chlorophyll content was determined by the method of Arnon (1949). An additional three leaf discs were extracted with 5 ml of cold Tris-HCl buffer (pH 6.8), after centrifugation (10,000 x g, 10 min), supernatant was precipitated with 80% cold acetone and stored overnight at -20°C. After washing three times with cold acetone, the precipitate was resuspended into buffer solution, and total protein was determined by the method of Lowry (1951). Bovine serum albumin (BSA) was used as a standard. Total protein content was expressed as mg g^{-1} of leaf fresh weight. Ascorbic acid content of the leaf discs was determined by the method of Loeffler and Ponting (1942) following 72 hr incubation. Three leaf discs were homogenized at high speed for about 5 minutes in 5 ml of 3% oxalic acid. Following centrifugation and dilution, 4.5 ml of 2,6- dichloroindophenol (sodium derivative) dye (13 mg l^{-1}) was added, the absorbance of the reaction solution at 510 nm was measured 30 seconds later. The ascorbic acid content was calculated as following:

[(Absorbance of the dye - absorbance of the sample) × slope of the standard solution + intercept] × dilution factor / fresh weight (g)

The result was expressed as ascorbic acid content mg g^{-1} of fresh weight.

2. Intact individual leaf study

Leaf incubation and symptoms of leaf blackening evaluation. After arrival from the field, six individual leaves were detached from 6 flower stems. Each leaf was placed into individual test tubes containing 5 ml water or 5% (w/v) sucrose

solution held in the dark or in 12 hr of light/dark cycle, photosynthetically active radiation (PAR) of 45 μmol m^{-2} s^{-1}, 20 to 22 °C, and relative humidity of 60 to 80%. The symptoms of leaf blackening were determined every other day.

Carbon dioxide evolution. Carbon dioxide evolution was measured every other day. An individual leaf was weighed and sealed in a test tube for 1 hr, and a 1 ml of sample gas from the test tube was injected into nitrogen gas flow that passed through an infrared analyzer. The result was expressed as CO_2 ml kg^{-1} hr^{-1}.

Leaf leakage. Individual green leaves and leaves with 10 to 30% of their area blackened, were washed with 1 ml of deionized water. The collected washing was analyzed by the Service Unit of the Department of Agronomy and Soil Science for potassium and sodium by atomic absorption spectrophotometer, and calcium and magnesium were analyzed by Inductively Coupled Plasma (ICP).

Polyphenol oxidase (PPO) Activity. Polyphenol oxidase activity was determined on green fresh leaves, individual leaves held in water under complete darkness with 10 to 30% blackened leaf area, and leaves held in sucrose (5%, w/v) under complete darkness. Soluble and total PPO activity was determined according to the method of Sherman *et al.* (1991) with modification. Leaves were washed with deionized water, blotted dry, frozen in liquid nitrogen and ground with a mortar and pestle to fine powder in the presence of liquid nitrogen (mid-vein not included). One gram of sample was mixed with 5 ml Tris-HCl buffer (pH 6.8) containing 1% (w/v) insoluble polyvinyl pyrrolidone (PVP). Soluble PPO extraction was carried out by extracting the samples with buffer only, while 5% (w/v) of sodium dodecyl

sulphate (SDS) was used to extract membrane-bond PPO. The extraction was carried out at 1°C for 30 min. The extracts were centrifuged at 10,000 x g for 10 min at 4°C, and the supernatant was used to determine PPO activity. The PPO activity was assayed spectrophotometrically at 490 nm, 0.01 ml of crude extract was added to 1 ml of 10 mM catechol, and the change in absorbance recorded for the first 5 min. One unit of enzyme activity was defined as a change of one absorbance unit per min at 490 nm and 25°C. Soluble PPO activity was the PPO activity detected in leaf extractions without SDS, and total PPO activity was the sum of soluble plus membrane-bond PPO activity.

Effects of reducing agents, metabolic and non-metabolic sugars. Individual leaves were placed in test tubes containing 5 ml of 5% (w/v) metabolic sugars (sucrose or glucose) or non-metabolic sugar (sorbitol). To test the effect of reducing agents on leaf blackening or browning, an individual leaf was inserted in a test tube containing 5 ml of ascorbic acid, diphenylamine, dithiothreitol, and sodium bisulfite solution (all 10 mM). All were incubated in complete darkness until the end of the experiment (15 days after harvest). The rate of leaf blackening was recorded every other day.

3. Flower stem study

Light compensation point determination. The light compensation point for leaf photosynthesis was determined with a LI-6000 Portable Photosynthesis System (Li-Cor, Nebraska, U.S.A.). Different photosynthetically active radiation (PAR)

intensities were achieved by varying the distance of a incandescent light source (1000 W) to the leaf surface. Light was passed through a water filter to prevent temperature increases. The temperature was in the range of 22 to 24°C. A leaf chamber (20 cm × 10 cm) was pre-equilibrated with fresh air for 5 min. and a portion of the leaf was inserted into this chamber. Photosynthesis rate was measured as carbon dioxide concentration (mg s^{-1} cm^{-2}) decreased during a period of time (ca. min.). Carbon dioxide level was measured with a Infrared gas analyzer connected to the leaf chamber. The result was expressed as CO_2 mg s^{-1} cm^{-2} and plotted against light levels. The light compensation point was determined as the light level at which net CO_2 exchange was zero.

Sugar content determination. Total sugar content in leaves attached to flower stems held in water or in 5% (w/v) sucrose was determined 2 days after harvest by the method of Dubois *et al.* (1956). Fresh tissue (2.5 g) was homogenized in 25 ml 95% ethanol. A portion of the sample (2.5 µl) was diluted with deionized water to 1 ml, and mixed with 25 µl of 80% w/w phenol (20 ml water, 80 gm phenol). Ten minutes after mixing, 2.5 ml of H_2SO_4 was added and mixed well. After standing for 30 min. at room temperature (22 to 24°C), the absorbance at 485 nm was measured. The concentration of total sugar in the sample was determined with dextrose as the standard. The result was expressed as total sugar content mg dextrose g^{-1} of fresh or dry weight.

Effects of light on flower vase life. Six flower stems were held in water under different white influroscence light levels, and vase life were determined. Flower

stems were held under 12 h of high light level (45 μmol m^{-2} s^{-1}), dim light just below light compensation point (7-9 μmol m^{-2} s^{-1}), very dim light (1 to 3 μmol m^{-2} s^{-1}), and complete darkness. Flower head and leaves conditions were evaluated every other day, and vase life was determined as 50% of leaf blackening and bracts moderately curled as described in the General Materials and Methods section.

Light and sucrose interaction. Flower stems held in water or solutions with 0.5%, 2.5%, 5.0%, and 7.5% (w/v) sucrose concentrations were placed under 12 h light (45 μmol s^{-1} m^{-2}) or complete darkness. Effects of light, and sucrose at different concentrations on preventing leaf blackening were examined. The days to 50% of leaf blackening was used to evaluate light and sucrose effect.

C. Results

1. Leaf disc study

Leaf discs floating in water became brown after 48 hours in continuous darkness, while leaf discs exposed to 12 h light/dark remained green. After 72 hours incubation in darkness, leaf discs became totally brown and appeared water-soaked. Under 12 hr light/dark condition, leaf discs floating on water after 72 hours showed signs of blackening around the edge of leaf discs while the center of the discs were still green.

Chlorophyll fluorescence Fv/Fm ratio was highest in the fresh leaf (0.49, Table 2.1) when compared to discs under 12 h light/dark conditions for 72 hours. The Fv/Fm ratio of light/dark leaf discs declined to 73% of original value after 72

Table 2.1. Chlorophyll fluorescence parameter of leaf discs floating in water for 72 hours under either 12 h light/dark (light level: 45 μmol m^{-2} s^{-1}) or in continuous darkness.

Treatment	Chlorophyll fluorescence[z]			
	Initial (I) (Fo)	Peak (P) (Fm)	P - I (Fv)	(P -I)/P (Fv/Fm)
Fresh leaf	72 ±6	140 ±14	68	0.49
Light (12 h)	61 ±9	96 ±18	35	0.36
Dark	84 ±14	86 ±14	2	0.02

[z]Data were means of 6 replicate ±SE.

hr incubation while leaves in continuous darkness declined to 4% of the fresh leaf. Sucrose and reducing agents treatments did not prevent the loss of chlorophyll fluorescence of leaf discs kept under continuous darkness (Table 2.2). The Fv/Fm ratios were near zero with 72 hours of incubation regardless of the treatment.

Browning of the bathing solution. Browning of the leaf disc bathing solution was observed following 12 hr incubation in all treatments. When measuring solution absorbance at 490 nm during the first 24 hr, there was no significant difference between solutions with leaf discs floating in water in the dark and those under 12 hr light/dark condition (Figure 2.1). Solutions from both treatments were slightly brown and reddish, probably resulting from the release of phenolic compounds from the cut area and their subsequent oxidation. Differences between treatments began 36 hours after incubation. The browning of the leaf disc bathing solution in continuous darkness continued to increase until the end of the experiment (Figure 2.1), while the rate of increase for leaf discs on the 12 h light/dark cycle was much slower. By the end of the experiment (72 hours after incubation), the absorbance of the bathing solution from leaf discs in continuous darkness was almost twice as high as the solution from leaf discs receiving 12 h light/dark.

Treatment with 5 mM $CaCl_2$ slowed the rate of browning of the bathing solution in the 12 h light/dark condition (Figure 2.1); however, $CaCl_2$ had no effect when the discs were held in continuous darkness (Figure 2.1). Sucrose at 2.5% did not prevent solution browning when compared to leaf discs in water and in continuous darkness (Figure 2.2). Reducing agent, ascorbic acid, diphenylamine,

Table 2.2. Chlorophyll fluorescence of leaf discs floating on different solutions for 72 hours and held under continuous darkness.

Treatment	Chlorophyll fluorescence[z]			
	Initial (I) (Fo)	Peak (P) (Fm)	P - I (Fv)	(P - I)/P (Fv/Fm)
Water	84 ±14	86 ±14	2	0.02
Sucrose (2.5%)	82 ±10	84 ±10	2	0.02
DPA (10 mM)	55 ±9	57 ±9	2	0.04
DTT (10 mM)	92 ±14	94 ±15	2	0.02
Ascorbic acid (10 mM)	110 ±14	114 ±14	4	0.04
Sodium bisulfite (10 mM)	101 ±7	104 ±7	3	0.03

[z]Data are the means of 6 replicates ±SE.

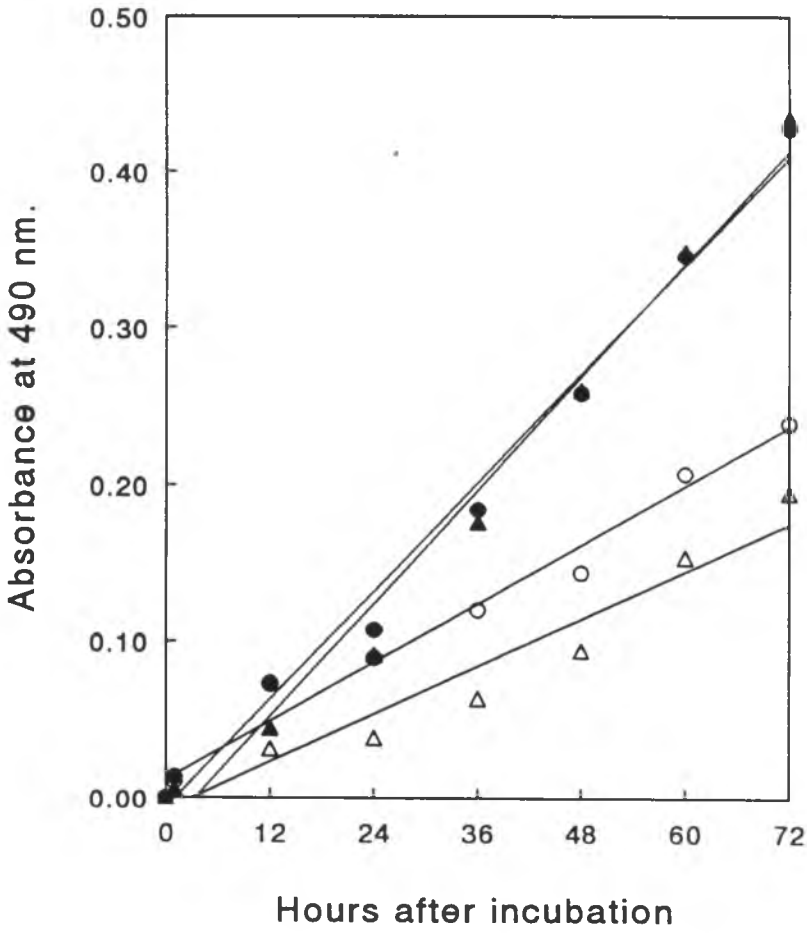
Figure 2.1. Absorbance of bathing solutions at 490 nm following incubation in water in 12 hr light/dark condition (o------o,) or in the dark (●------●), in 5 mM $CaCl_2$ solution in 12 h light/dark condition (▵------▵) or in the dark (▲------▲).

Regression analysis:

Treatments	Equation[z]	r^2
Water, 12 hr light/dark	$Y_1 = 11\times10^{-3} + 3.1\times10^{-3}$ X	0.99***[y]
Water, dark	$Y_2 = -6.6\times10^{-3} + 5.8\times10^{-3}$ X	0.99 ***
5 mM $CaCl_2$, 12 hr light/dark	$Y_3 = -7.8\times10^{-3} + 2.5\times10^{-3}$ X	0.97**
5 mM $CaCl_2$, dark	$Y_4 = -20\times10^{-3} + 6\times10^{-3}$ X	0.99***

[z]Y = predicted value of absorbance at 490 nm; X = hours of incubation.

[y]**, ***, significance at 1% and 0.1% levels respectively.


0.50
0.40
0.30
0.20
0.10
0.00
Absorbance at 490 nm.
0
12
24
36
48
60
72
Hours after incubation

dithiothreitol, and sodium bisulfite, did reduce the degree of leaf disc browning and prevented solution browning. The bathing solutions treated with reducing agents all had very low 490 nm absorbance throughout the experimental period (Figure 2.3).

Chlorophyll, total protein, and ascorbic acid content. Leaf disc chlorophyll, total protein, and ascorbic acid contents were compared when bathed in water for 72 hr and kept either under 12 h light/dark or continuous darkness. Fresh leaves were used as the control. The chlorophyll content of leaf discs bathing in water in continuous darkness was significantly lower than that of both fresh leaves and leaf discs floating in water under 12 h light/dark (Figure 2.4 A). There was no significant difference in chlorophyll content between fresh leaf and leaf discs floating in water and kept under 12 light/dark condition (Figure 2.4 A). Total protein content in leaf discs floating in water and kept under 12 h light/dark was significantly lower than those found in fresh leaves, but significantly higher than those found in leaf discs floating in water and in continuous darkness (Figure 2.4 B). Ascorbic acid in leaf discs floating in water under 12 light/dark was significantly lower than those found in the fresh leaves (Figure 2.4 C). Leaf discs bathing in water and kept under continuous darkness had the lowest ascorbic acid, being only 10% of that in fresh leaves and 20% of that in leaf discs floating in water under 12 h light/dark (Figure 2.4 C).

2. Intact individual leaf study

Carbon dioxide evolution. Carbon dioxide evolution from leaves held in

Figure 2.2. Absorbance of bathing solutions at 490 nm following incubation of leaf discs in water held under 12 hr light/dark condition (o------o) or in the dark (●------●), and in 2.5% (w/v) sucrose in the dark (▵------▵).

Regression analysis:

Treatments	Equation[z]	r^2
Water, 12 hr light/dark	$Y_1 = 11 \times 10^{-3} + 3.1 \times 10^{-3} X$	0.99***[y]
Water, dark	$Y_2 = -6.6 \times 10^{-3} + 5.8 \times 10^{-3} X$	0.99***
2.5% sucrose, dark	$Y_3 = -8.6 \times 10^{-3} + 6.1 \times 10^{-3} X$	0.99***

[z]Y = predicted value of absorbance at 490 nm; X = hours of incubation.

[y]**, ***, significance at 1% and 0.1% levels respectively.

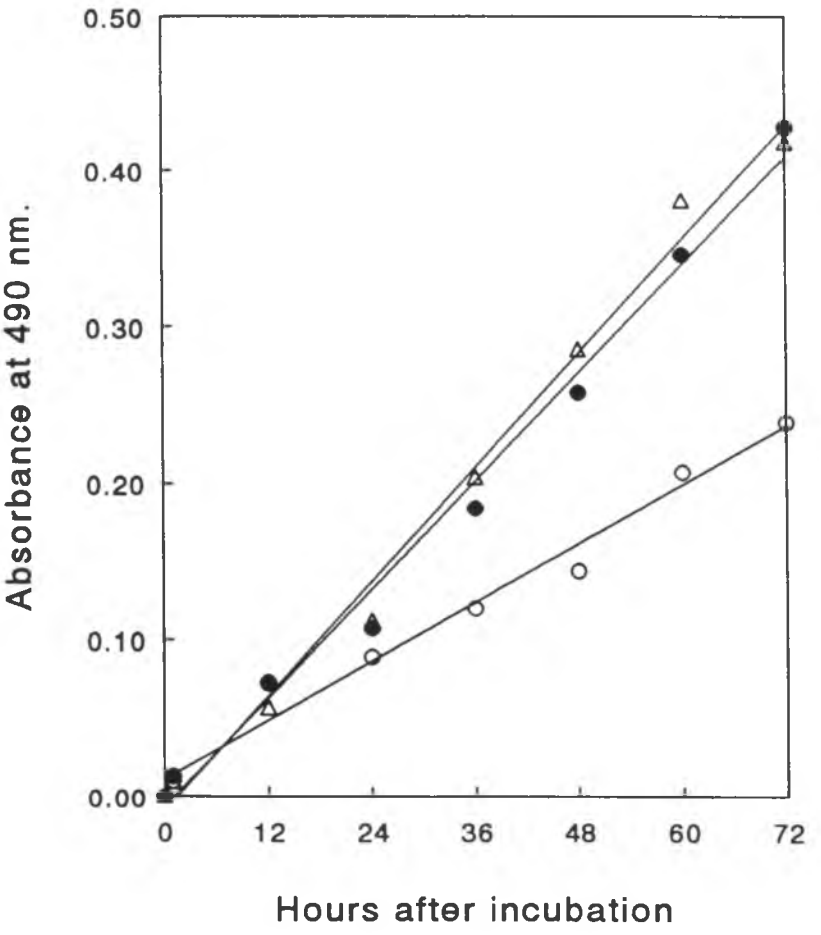

0.50
0.40
0.30
0.20
0.10
0.00
Absorbance at 490 nm.
0
12
24
36
48
60
72
Hours after incubation

Figure 2.3. Absorbance of bathing solutions at 490 nm following incubation of leaf discs in water either held under 12 hr light/dark condition (o------o) or in the dark (●-----●). In the dark, +------+, 10 mM DTT; ▲------▲, 10 mM DPA; ◇------◇, 10 mM ascorbic acid; and □------□, 10 mM SBS.

Regression analysis:

Treatments	Equation[z]	r^2
Water, 12 hr light/dark	$Y_1 = 11\times10^{-3} + 3.1\times10^{-3}$ X	0.99***[y]
Water, dark	$Y_2 = -6.6\times10^{-3} + 5.8\times10^{-3}$ X	0.99***
In the dark, 10 mM DTT	$Y_3 = 4.8\times10^{-3} + 0.1\times10^{-3}$ X	0.42 [ns]
10 mM DPA	$Y_4 = -15.6\times10^{-3} + 5.7\times10^{-3}$ X	0.99***
10 mM ascorbic acid	$Y_5 = -0.3\times10^{-3} + 0.7\times10^{-3}$ X	0.91**
10 mM SBS	$Y_6 = 4.7 \times 10^{-3} + 0.04\times10^{-3}$ X	0.2 [ns]

[z]Y = predicted value of absorbance at 490 nm; X = hours of incubation.

[y]ns, **, ***, non-significant, significance at 5%, 1%, and 0.1% levels respectively.

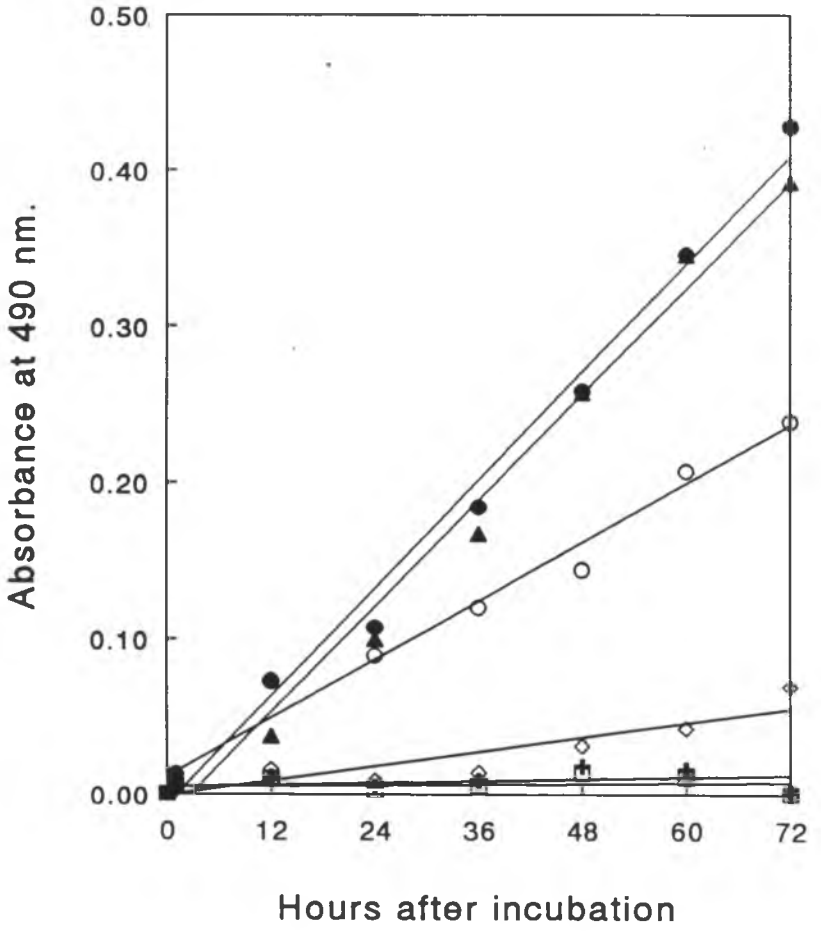
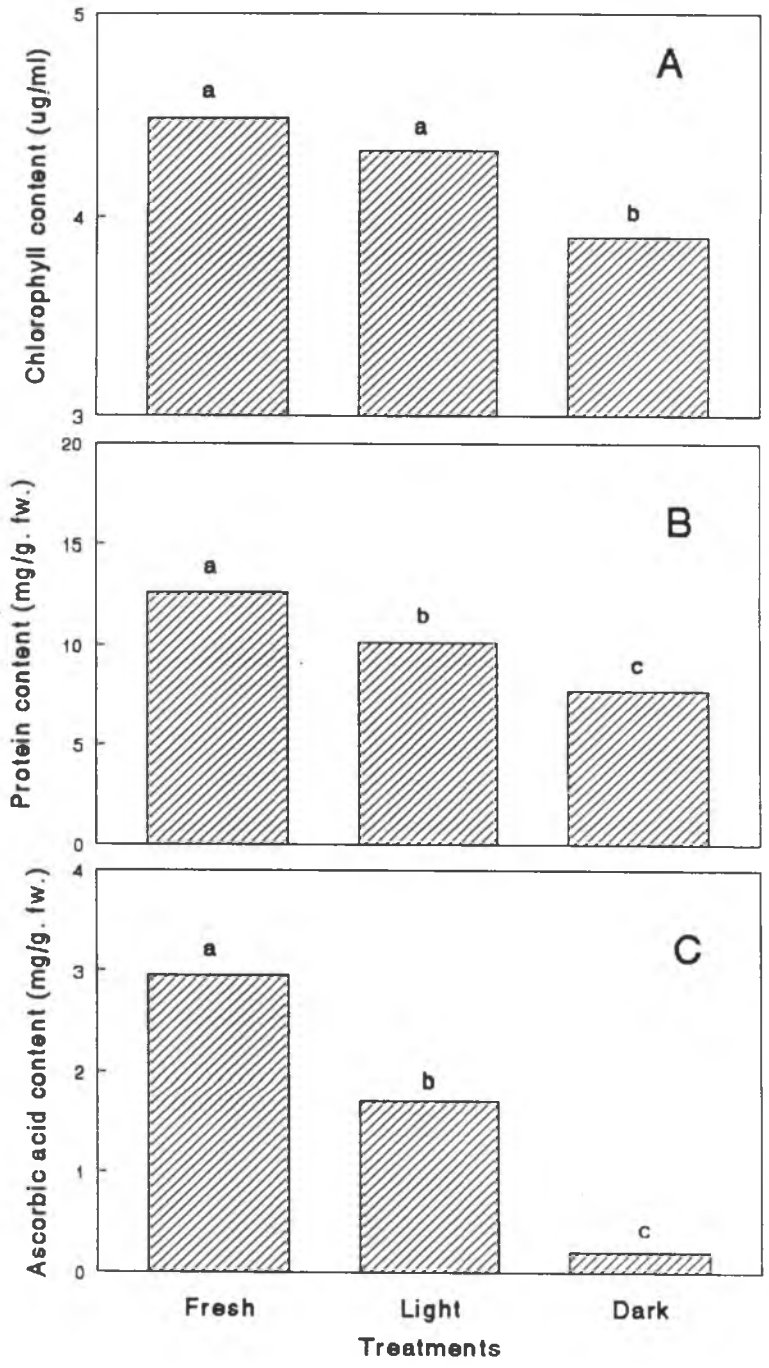

0.50
0.40
0.30
0.20
0.10
0.00
Absorbance at 490 nm.
0
12
24
36
48
60
72
Hours after incubation

Figure 2.4. Chlorophyll (A), total protein (B), and ascorbic acid (C) content of leaf discs bathing in water under 12 h light/dark, or in the dark, compared to fresh leaves. Mean separation by Duncan-Waller multiple range test ($P<0.01$, $n = 5$), bars with the same letter are not significantly different.


A
Chlorophyll content (ug/ml)
5
4
3
a
a
b
B
Protein content (mg/g. fw.)
20
15
10
5
0
a
b
c
C
Ascorbic acid content (mg/g. fw.)
4
3
2
1
0
a
b
c
Fresh
Light
Dark
Treatments

water in 12 hr light/dark during the experiment was significantly lower than those held in darkness (Figure 2.5). Under light, the carbon dioxide evolution per leaf was 27 ml kg^{-1} hr^{-1}, 2 days after harvest, and then declined. Eight days after harvest, the carbon dioxide evolution declined to the lowest level, 10 ml kg^{-1} hr^{-1}, about 30% of that 2 days after harvest.

Carbon dioxide evolution from leaves held in complete darkness was 90 ml kg^{-1} hr^{-1}, 2 days after harvest, 3 times higher than those found in leaves held in light (Figure 2.5). Eight days after harvest, the level of carbon dioxide evolution had declined to 65 ml kg^{-1} hr^{-1}, a 25% decease compared to that in leaves 2 days after harvest. The carbon dioxide level continued to decline to the lowest level 14 days after harvest (56 ml kg^{-1} hr^{-1}), and then rose to 70 ml kg^{-1} hr^{-1} 16 days after harvest (Figure 2.5).

Leaves held in sucrose (5%, w/v) in complete darkness had the highest carbon dioxide evolution rates (Figure 2.5), although there was no significant difference in carbon dioxide evolution between leaves held in water and leaves held in sucrose under complete darkness 2 days after harvest. There was a decline in carbon dioxide evolution in leaves held in sucrose, from 100 ml kg^{-1} hr^{-1} to about 90 ml kg^{-1} hr^{-1}, 14 days after harvest.

Leaf condition. Leaves held in water under 12 hr light/dark or in sucrose (5%, w/v) in complete darkness did not turn black during the 16 days experimental period. Leaves held in water and in complete darkness began to turn black (about 10% of the leaf area) 10 days after harvest (Figure 2.5). The blackening appeared

Figure 2.5. Carbon dioxide evolution of leaves held either in water under 12 h light/dark (o------o) or in the dark (●------●), and of leaves held in sucrose (2.5% w/v) in the dark (▵-----▵).

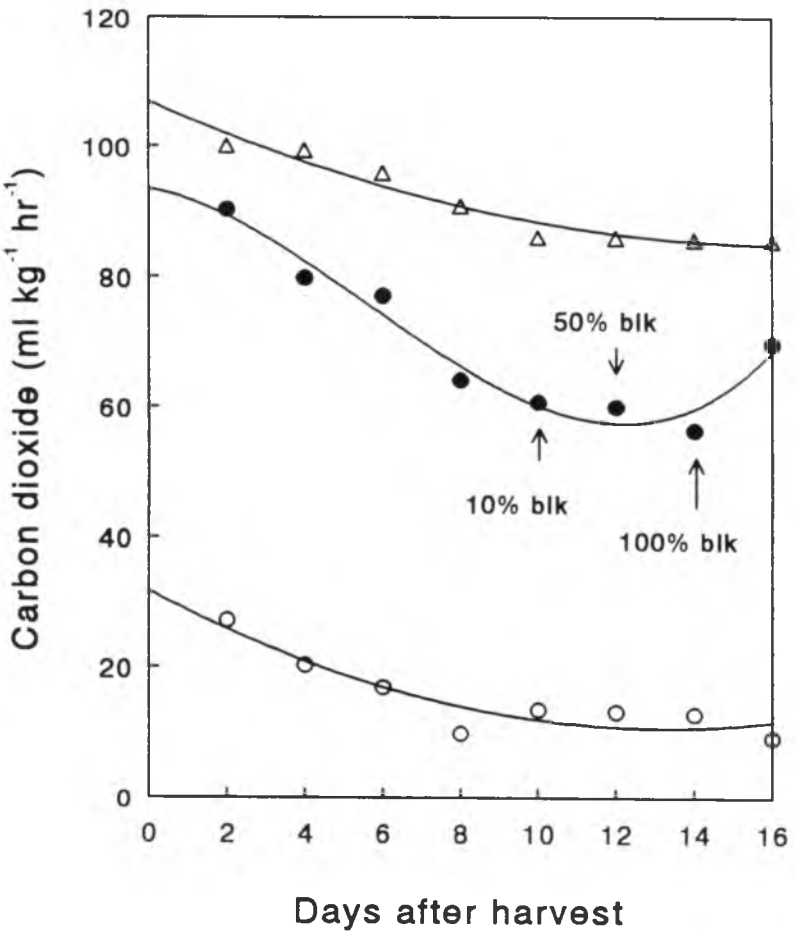
Regression analysis:

Treatments	Equation[z]	r^2
Water, 12 hr light/dark	$Y_1 = 31.74 - 3.2X + 0.12X^2$	0.92**[y]
Water, dark	$Y_2 = 93.49 - 1.12X - 0.54X^2 + 0.032X^3$	0.98***
5% sucrose, dark	$Y_3 = 106.91 - 2.66X + 0.08X^2$	0.97***

[z]Y = predicted value of carbon dioxide evolution (ml kg^{-1} hr^{-1}), X = days after harvest.

[y]**, ***, significance at 1% and 0.1% levels respectively.

Abbreviation: Blk = Leaf blackening.


120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
Carbon dioxide (ml kg-1 hr-1)
Days after harvest
50% blk
10% blk
100% blk

as small spots on the leaf surface or at the leaf edges. Black leaf exudate was observed on the blackened area, and when the exudate dried out, the blackened leaf area became sunken and dry. The small black spots coalesced to form a larger black area, and 12 days after harvest, 50% of the leaf area had become black. The blackening rate increased, and the leaves became 100% black 14 days after harvest.

Leachate analysis. Leaves held in water in complete darkness had 10% to 30% blackened leaf area 10 to 12 days after harvest. A black exudate was observed on the surface of the blackened area. No black exudate was observed on the surface of leaves held in water under 12 hr light/dark, or in 5% sucrose in complete darkness. Leachate from the 10 to 30% blackened leaves held in water under complete darkness for 10 to 12 days contained almost 3 times higher Ca, 4 times Na, and 5 times K and Mg than from leaves held in water in 12 hr light/dark or leaves in 5% (w/v) sucrose in the dark (Table 2.3).

Polyphenol oxidase (PPO) activity. Soluble PPO activity in leaves held in water under 12 hr light/dark or held in 5% (w/v) sucrose solution under complete darkness was only about 0.4 units (Table 2.4). However, PPO activity increased after extraction with SDS. Total PPO activity in leaves of these two treatments was about 14 units, 35 times higher than soluble PPO activity. The proportion of soluble PPO activity was only 3% of total PPO activity. Soluble PPO activity increased about 3-fold in 10 to 30% blackened leaves compared to the green leaves (Table 2.4), while total PPO activity decreased to half of those found in green leaves. In these partially blackened leaves, the proportion of soluble PPO activity increased, and was 16% of

Table 2.3. Analysis of leaf exudates from the leaves held in water under 12 h light/darkness (PAR: 45 μmol m^{-2} s^{-1}) or in continuous darkness, and leaves held in 5% (w/v) sucrose in the dark 10 days after harvest.

Treatments	Elements in leaf exudates[z] (ppm)			
	K	Ca	Mg	Na
12 hr light/dark	2.74 ±0.34.	5.20 ± 0.43	2.0 ± 0.11	2.13 ± 0.15
Dark	12.9 ±0.48	14.0 ± 0.45	10.4 ± 0.23	8.66 ± 0.26
Sucrose in dark	2.68 ±0.31	5.13 ± 0.41	2.08 ± 0.18	2.11 ± 0.15

[z]Data means of 3 replicates, ±SE.

Table 2.4. Soluble and total polyphenol oxidase activities of individual leaves held either in water under 12 hr light/dark (45 μmol m^{-2} s^{-1}) or in the dark, and leaves held in 5% (w/v) sucrose in the dark. Activity was assayed when the leaves held in the dark in water showed 10 to 30% blackening, about 10 to 12 days after harvest.

Treatments	PPO activity (units ml^{-1})[z]		Ratio
	Soluble	Total	Soluble/Total
Light/dark	0.40 ±0.12	13.84 ±0.61	0.03
Dark	1.26 ±0.13	7.76 ±0.55	0.16
Sucrose in dark	0.34 ±0.11	14.42 ±0.65	0.03

[z]Data means of 3 replicates, ±SE.

the total PPO activity. The ratio of soluble to total PPO activity (the disassociation ratio) in partially blackened leaves was more than 5 times higher than green leaves (Table 2.4).

Effect of reducing agents, metabolic and non-metabolic sugar on leaf blackening. The reducing agents, ascorbic acid, diphenylamine, dithiothreitol, sodium bisulfite and the non-metabolic sugar sorbitol, did not delay or prevent individual leaf blackening under dark condition (Table 2.5). Fifteen days after harvest, 80 to 100% of leaves had become black. Metabolic sugars such sucrose, glucose, and 12 hr light/dark conditions prevented leaf blackening (Table 2.5), with no sign of blackening 15 days after harvest.

3. Flower Stem Study

Light compensation point. The net photosynthesis rate (NPR) increased as photosynthetically active radiation (PAR) level increased (Figure 2.6). The NPR was negative when PAR was near zero. When PAR was about 10 μmol m^{-2} s^{-1}, the NPR was zero, this was considered as the light compensation at 22°C, and 60% RH. There was a positive linear relationship between NPR and PAR, when PAR was in the range from zero to 220 μmol m^{-2} s^{-1} (Figure 2.6). The light saturation point was not within this PAR range.

Vase life under different light level. Flower stems held in water under darkness or at very low PAR levels (1 to 3 μmol m^{-2} s^{-1}) had the shortest postharvest life (around 10 days) for both bracts and leaves (Figure 2.7). Although at PAR levels

Table 2.5. Effect of continuous darkness, 12 hr light, reducing agents, metabolic and non-metabolic sugar on individual leaf blackening. Leaf condition was evaluated 15 days after harvest.

Treatments	Leaf condition (% blackening)[z]
Control (darkness)	85 b
Light (45 μmol m^{-2} s^{-1}, 12 hr)	0 c
Sucrose 5% (w/v)	0 c
Glucose 5% (w/v)	0 c
Sorbitol 5% (w/v)	80 b
Ascorbic acid (10 mM)	86 b
Diphenylamine (10 mM)	100 a
Dithiothreitol (10 mM)	100 a
Sodium bisulfite (10 mM)	100 a

[z]Data were analyzed by Duncan-Waller multiple range test, means followed by the same letter were not significantly different ($P<0.05$, $n = 10$).

Figure 2.6. Relationship between net photosynthesis rate (NPR) of leaves on flower stems held in water and levels of photosynthetically active radiation (PAR), at 20 to 22 °C, 60 to 80% RH. NPR = -0.11 + 0.011 PAR, $r^2 = 0.97$, $P<0.001$.

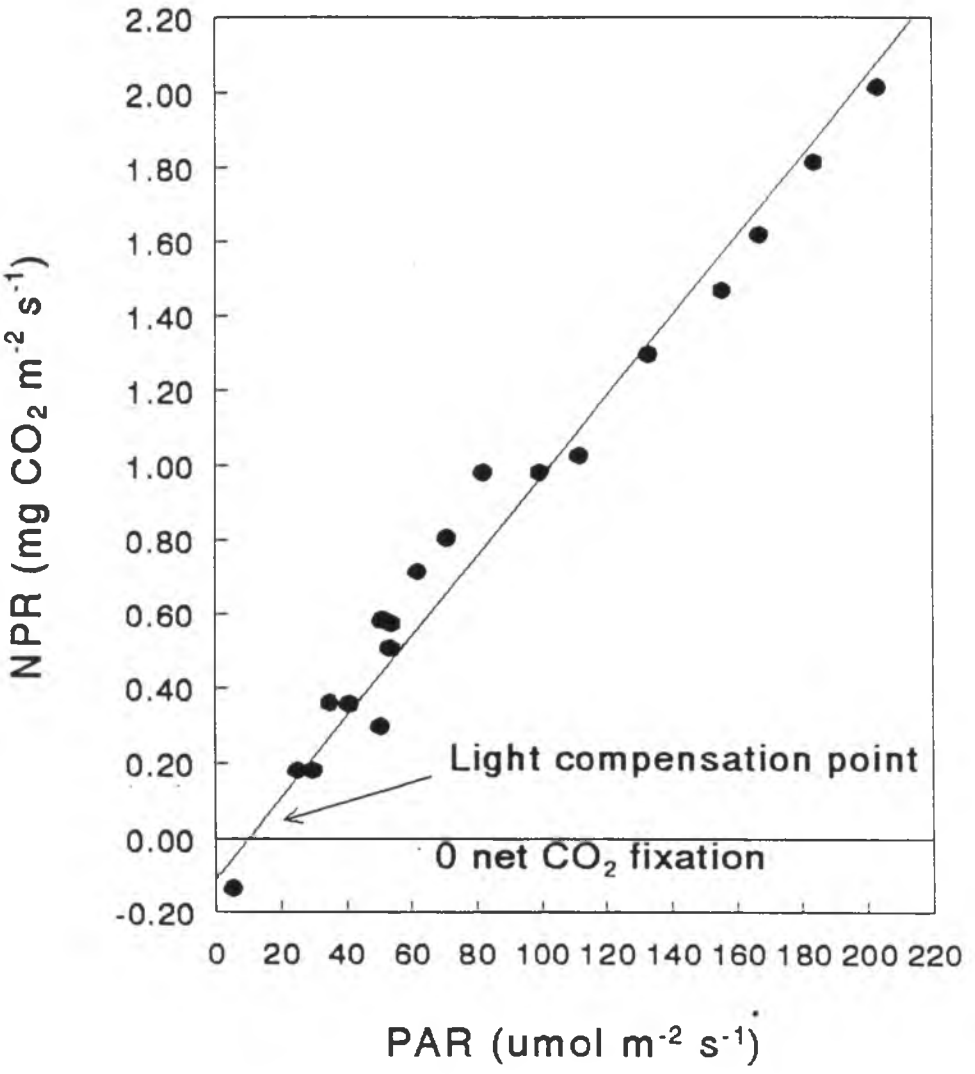

2.20
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
-0.20
NPR (mg CO_2 m^{-2} s^{-1})
Light compensation point
0 net CO_2 fixation
0 20 40 60 80 100 120 140 160 180 200 220
PAR (umol m^{-2} s^{-1})

Figure 2.7. Postharvest life of flower and leaves on flower stems held in deionized water under different light levels. o------o, inner bracts; ●------●, outer bracts; ▵-----▵, leaves.

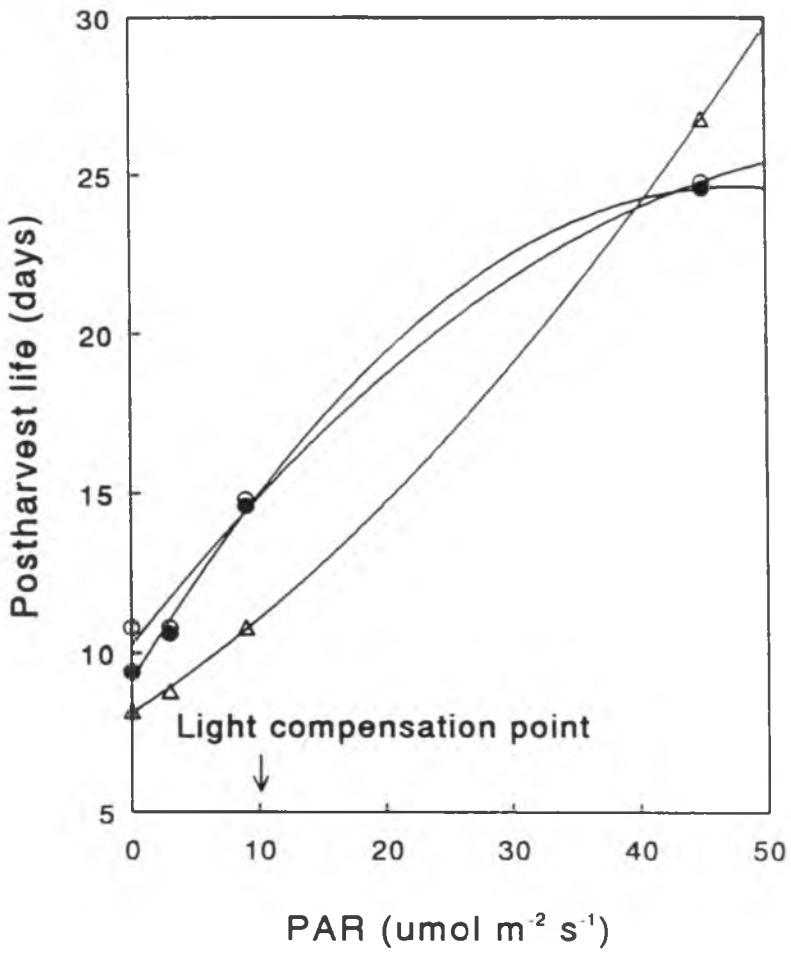
Regression analysis:

Predicted postharvest life of	Equation[z]	r^2
Inner bracts	$Y_1 = 9.91 + 0.87X - 0.01X^2$	0.98***[y]
Outer bracts	$Y_2 = 9.13 + 0.66X - 0.008X^2$	0.99***
Leaves	$Y_3 = 8.12 + 0.26X + 0.003X^2$	0.99***

[z]Y_1, Y_2, Y_3, predicted postharvest life of inner bracts, outer bracts, and leaves.

X = Photosynthetically active radiation (PAR).

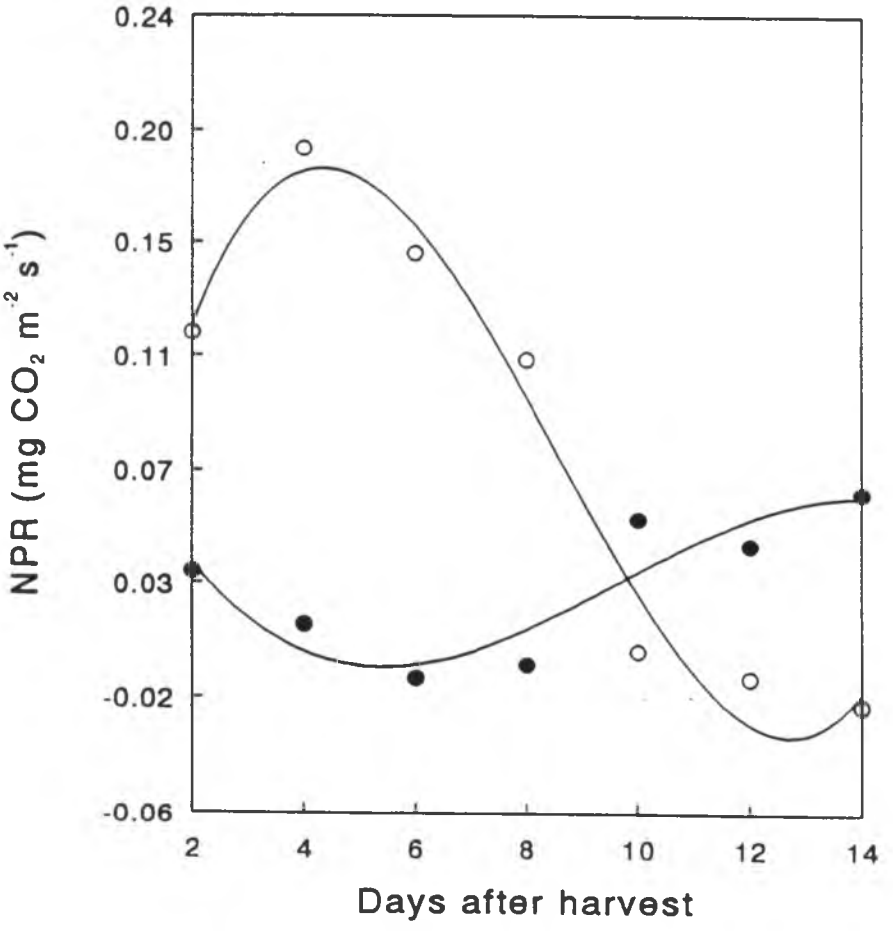
[y]*** significance at 0.1%, n = 6.


30
25
20
15
10
5
Postharvest life (days)
Light compensation point
0
10
20
30
40
50
PAR (umol m-2 s-1)

of 7 to 9 μmol m^{-2} s^{-1} there was significant improvement in bract postharvest life (around 15 days), this light level did not improve leaf postharvest life significantly (Figure 2.7). Flower stems kept at 45 μmol m^{-2} s^{-1} PAR had significantly longer postharvest life as judged by bract condition and leaf blackening than stems held at lower PAR levels. The postharvest life of flower bracts on the stems kept at 45 μmol m^{-2} s^{-1} was double that of those held in darkness or lower light condition, and 35% longer than that of those held just below light compensation point. There was no significant difference in leaf postharvest life among flower stems held below the light compensation point level. However, the postharvest life of leaves still attached to the flower stems kept at 45 μmol m^{-2} s^{-1} was three times greater than those held in the dark or at very low light condition, and 2.5 times greater than those held below the light compensation point. There were trends of postharvest life improvement as light level increased (Figure 2.7).

Sucrose treatment, leaf NPR and leaf blackening. Net photosynthesis rate (NPR) of leaves attached to flower stems held in water or in 5% (w/v) sucrose was compared. The stems were held in dim light (20 to 22 μmol m^{-2} s^{-1} PAR), at 20 to 22°C and 60 to 80% RH. The NPR of leaves attached to the flower stem held in water was initially high, ca. 0.12 mg CO_2 m^{-2} s^{-1}, 2 days after harvest (Figure 2.8). This rate increased to 0.19 mg CO_2 m^{-2} s^{-1}, 4 days after harvest, the highest NPR detected. The NPR declined gradually to the initial level 8 days after harvest, then sharply declined to zero within 2 days. No positive NPR was detected 10 days after harvest.

Figure 2.8. Net photosynthesis rate (NPR) of leaves attached to flower stems and held in 12 hr light/dark condition either in water (o-----o) or 5% (w/v) sucrose solution (●-----●). Light level was 22 μmol m^{-2} s^{-1}. Leaf NPR in water: $Y_1 = - 0.05 + 0.12X - 0.019X^2 + 0.7 \times 10^{-3}X^3$, $r^2 = 0.98^{***}$; in sucrose (5%, w/v): $Y_2 = 0.105 - 0.047X + 0.006X^2 - 0.21 \times 10^{-3}X^3$, $r^2 = 0.90^{**}$. X = days after harvest. **, ***, significance at 1%, and 0.1% levels respectively, n = 5.


0.24
0.20
0.15
0.11
0.07
0.03
-0.02
-0.06
NPR (mg CO_2 m^{-2} s^{-1})
2
4
6
8
10
12
14
Days after harvest

Sucrose (5% w/v) significantly lowered NPR. The initial NPR of leaves attached to the flower stems treated with 5% sucrose was only 0.03 mg m^{-2} s^{-1}, 2 days after harvest (Figure 2.8), one fourth of that of leaves attached to flower stem held in water. The rate continued to decline, with ca zero NPR 6 to 8 days after harvest. However, NPR began to increase 8 days after harvest, and reached to 0.05 mg m^{-2} s^{-1} within 2 days. The NPR remained constant at this level till the end of the experiment, 14 days after harvest (Figure 2.8).

Leaves attached to flower stems held in water had a lower sugar content than those held in 5% (w/v) sucrose (Table 2.6). Sugar content in leaves of flower stems held in water was 50 mg g^{-1} of fresh weight, 2 days after harvest, while leaves of flower stems treated with 5% sucrose was 117 mg g^{-1}.

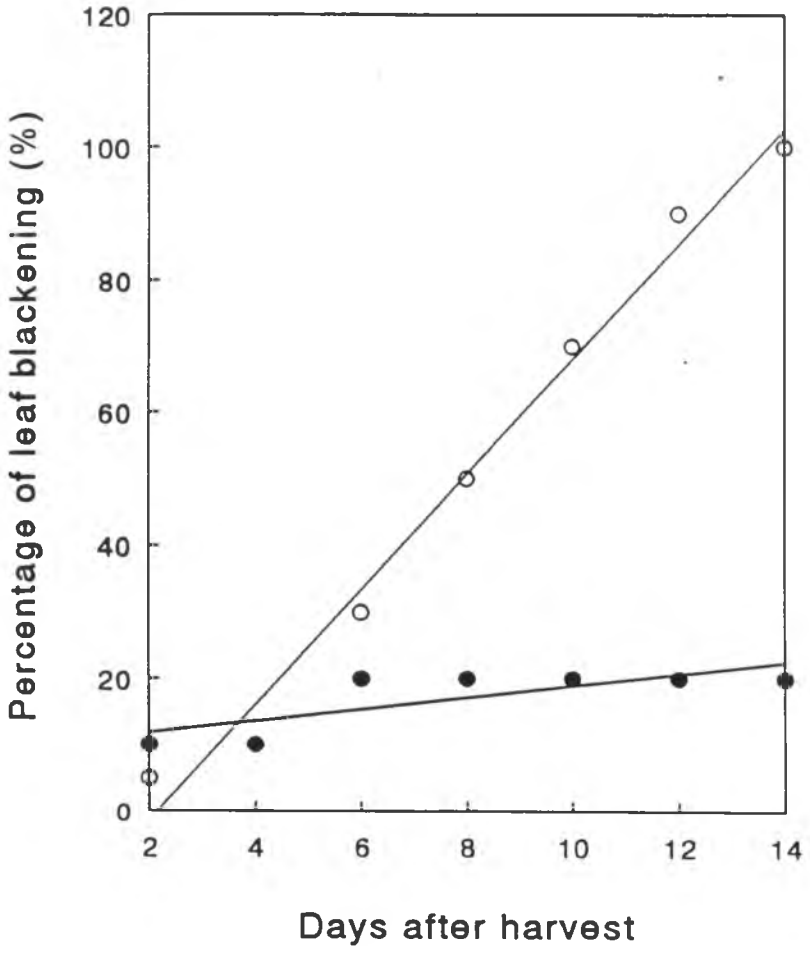
Leaves attached to flower stems held in water began to show blackening 2 days after harvest (Figure 2.9). The blackening appeared first at the leaf edge and gradually developed inward. Four days after harvest, 10% of leaf area had become black. The increase of blackening rate was linear, 10% per day, 50% of leaf area was black 8 days after harvest, and leaves became completely blackened 14 days after harvest. Leaves of the sucrose (5% w/v) treated flower stems initially showed some leaf discoloration at the leaf edges that was different from leaf blackening disorder. This discoloration was grey to light brown, and stabilized 6 days after harvest (Figure 2.9). Blackening did not occur during the experiment when the flower stems were treated with 5% sucrose.

Table 2.6. Total sugar content as dextrose of leaves attached on flower stems held either in water or in 5% (w/v) sucrose under 22 μmol m^{-2} s^{-1} PAR for 2 days.

Treatments	Sugar content (mg g^{-1})[z] (dextrose equivalent)	
	Fresh weight	Dry weight
Water	50.6 ±7.7	122.0 ±9.9
5% sucrose	117.5 ±20.3	248.7 ±46.1

[z]Data are means of 5 replicates, ±SE.

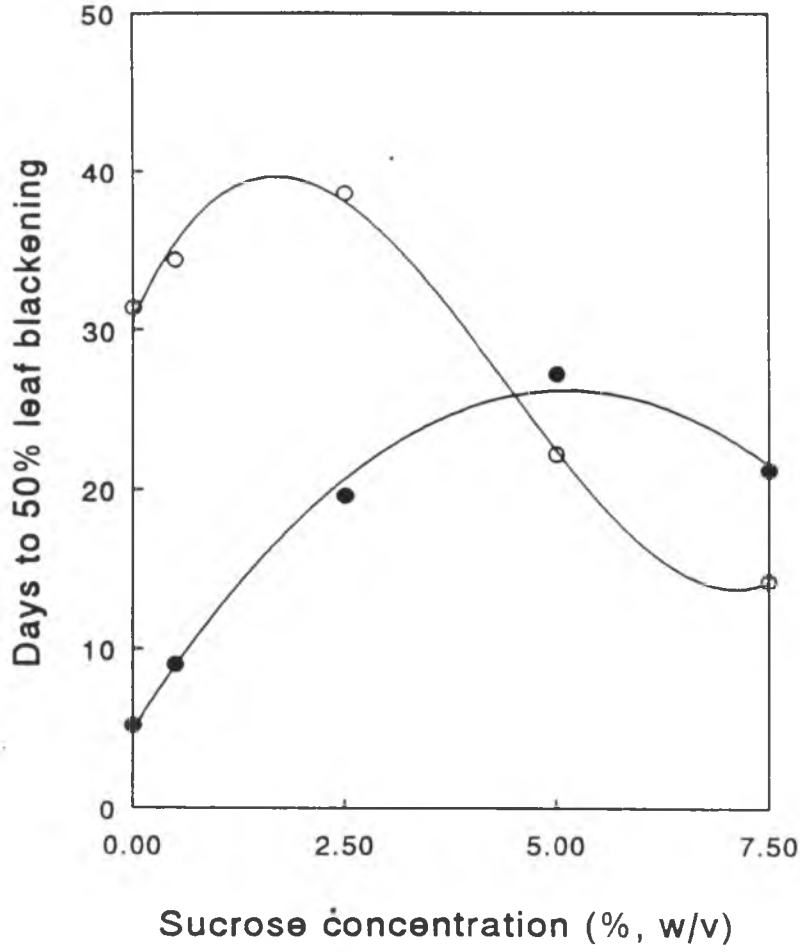
Figure 2.9. The rate of leaf blackening attached to flower stems held under 12 hr light/dark condition either in water (o-----o), or in 5% (w/v) sucrose solution (●-----●). Light level: PAR = 22 μmol m^{-2} s^{-1}. The rate of leaf blackening on flower stems held in water: Y_1 = - 18.57 + 8.66X, r^2 = 0.99***; in sucrose: Y_2 = 10 + 0.89X, r^2 = 0.79**. X = days after harvest. **, ***, significant at 1%, and 0.1% levels respectively, n = 5.


Percentage of leaf blackening (%)
120
100
80
60
40
20
0
2
4
6
8
10
12
14
Days after harvest

Effect of light, sucrose and their interaction on delaying leaf blackening. Leaves on flower stems held in water under 12 h light (45 μmol m^{-2} s^{-1}) had longer postharvest life than those under complete darkness (Figure 2.10). In complete darkness, leaf blackening occurred 3 days after harvest. The blackening of the leaves appeared first at the edge of the leaf, and then spread towards the midrib. Small black spots were also observed on some of the leaves surface, and black leaf exudate was found on the surface of these small black spots. As the number of these small black spots increased they coalesced to form larger blackened areas, and then dried out. Five days after harvest, 50% of the leaf area on the flower stem became blackened (Figure 2.10), and 100% 8 days after harvest.

In 12 h light/dark condition, the days from harvest to 50% of leaves blackened increased to 30 days, a 6 fold increase (Figure 2.10). Under 12 h light/dark condition, a 2.5% sucrose seemed to be the best overall for keeping leaves from blackening (Figure 2.10). Concentrations higher than 5% showed signs of toxicity that significantly reduced the days needed for the leaves to reach 50% blackening. However, in complete darkness, sucrose concentration had an effect in delaying leaf blackening (Figure 2.10). Sucrose at 0.5% (w/v) in the dark did not delay leaf blackening, but as the sucrose concentration increased the days needed for leaves to reach 50% blackening also increased (Figure 2.10). The days to become 50% leaf blackening increased to 20 days when sucrose concentration was 2.5%, and 27 days when sucrose was 5%, 4- and 5-fold increases over no sucrose, respectively. However, further increase in sucrose concentration led to toxicity, and caused a

Figure 2.10. Days to 50% leaf blackening of leaves attached to flower stems held in various sucrose concentration (w/v) either in 12 hr light/dark condition (o-----o), or in the dark (●-----●). The light level was 45 μmol m^{-2} s^{-1}. Days to 50% leaf blackening of leaves on flower stems held in 12 hr light/dark cycle: $Y_1 = 30.69 + 11.6X - 4.27X^2 + 0.32X^3$, $r^2 = 0.99^{***}$; in the dark: $Y_2 = 4.94 + 8.32X - 0.81X^2$, $r^2 = 0.99^{***}$. X = sucrose concentration. **, *** significant at 1%, 0.1% respectively, n = 6.


50
40
30
20
10
0
Days to 50% leaf blackening
0.00
2.50
5.00
7.50
Sucrose concentration (%, w/v)

decline in leaf longevity. The optimum concentration of sucrose for delaying leaf blackening in complete darkness was 5% (Figure 2.10).

D. Discussion

1. Leaf disc study

Darkness induced leaf senescence has been reported in many important economic crops studies (Goldthwaite and Laetscg 1967; Thimann *et al.*, 1977; Wittenbach, 1977). Protein and chlorophyll decline have been used as indicators of leaf senescence (Thomas and Stoddart, 1980). In recent years, chlorophyll fluorescence emission from plant leaves has been used as a method in studying and monitoring photosynthetic events and judging the physiological state of the plant (Krause and Weis, 1991). The ratio of Fv/Fm, calculated as (Fm-Fo)/Fm, has become an important and easily measurable parameter of the physiological state of the photosynthetic apparatus in plant leaves. (Fo and Fm are measures of the instantaneous fluorescence and the maximum fluorescence that result from the first saturating flash measured in dark-adapted leaves). Environmental stresses that affect PS II efficiency lead to a characteristic decrease in Fv/Fm (Krause and Weis, 1991).

In the present study, a leaf disc floating on water under complete darkness became brown after 72 hr incubation. It had significantly lower chlorophyll and protein content than leaf discs under 12 h light/dark condition for the same period of time (Figure 2.4 A, B). The ratio of Fv/Fm decreased almost to zero in leaf discs floating in water under complete darkness for 72 hours (Table 2.1). Leaf discs

floating on water under 12 h light/dark remained green, had significantly higher chlorophyll and total protein content (Figure 2.4 A, B), and higher Fv/Fm ratio (Table 2.1), indicating that the chloroplasts were photosynthetically active. The leaf discs bathing solution under complete darkness browned quickly as measured by 490 nm absorbance (Figure 2.1). The browning of bathing solution was probably caused by leakage of phenolic substance from the leaf tissues as a result of membrane damage, and subsequent oxidation. Light may have a protective effect on the membrane system and therefore prevent leakage, since the bathing solution browness under 12 hr light/dark was 50% lower than that under complete darkness (Figure 2.1).

Calcium has been reported as having an effect on preventing membrane damage (Thompson, 1988) and therefore should reduce the degree of browning of the bathing solution. This reaction to 5 mM $CaCl_2$ was found only under the 12 hr light/dark condition (Figure 2.1). In complete darkness, no significant difference in browning was observed between $CaCl_2$ treated and non-treated leaf bathing solutions (Figure 2.1).

Sucrose has been reported to have the capacity of protecting cell membrane *in vitro* (Coorts, 1973; Parups and Chan, 1973), therefore it is included in some buffer systems to protect membrane integrity. However, in the present study, 2.5% (w/v) sucrose did not prevent leaf discs from browning, and the bathing solution browness was not different from that of leaf discs floating in water in the darkness (Figure 2.2). The Fv/Fm ratio of leaf discs floating on sucrose solution under

complete darkness was close to zero (Table 2.2), indicating loss of photosynthetic activity.

Reducing agents such as ascorbic acid are used to prevent fruit browning during processing (Labuza and Schmidl, 1986). Ascorbic acid level in plant tissue has been considered as a factor determining the tissue browning potential of banana (Jayaraman *et al.*, 1982), avocado (Kahn, 1975; Van Lelyveld and Bower, 1984; Van Lelyveld *et al.*, 1984; Bower and Van Lelyveld, 1985), and mango (Joshi and Shiralkar, 1977; Venkaiah and Patwardhan, 1977). Ascorbic acid prevents polyphenol oxidation (Labuza and Schmidl, 1986). Use of sodium bisulfite to prevent food browning in the food industry is now limited (Labuza and Schmidl, 1986; Anon, 1986). Bisulfite has been described as a PPO inhibitor, but it also react with phenolics to prevent tissue browning (Labuza and Schmidl, 1986). Dithiothreitol (DTT) is a reducing agent containing -SH HS- group used to prevent enzyme oxidation (Halliwell, 1984). Diphenylamine (DAP), another reducing agent, has been reported to delay *Protea* leaf blackening, though no data was presented (Jones and Clayton-Greene, 1991). When all the above reducing agents were tested (10 mM) in the dark, all discs showed significant reduction in bathing solution browning (Figure 2.3), and leaf discs floating in these solutions were visibly yellow, not brown. However, they did not prevent the decrease of Fv/Fm ratio (Table 2.2), indicating that the loss of photosynthetic activity of the leaf cells still occurred.

Leaf discs have been used to study leaf senescence in many species (Zrenner and Stitt, 1991). It is easy to establish short-term stressed condition. However, leaf

discs study may not be suitable for *Protea* leaf study, since protea leaf discs turned brown or black around the cut wounding area, and floating solutions easily darken even when the leaf discs were cut under reducing condition (i.e. ascorbic acid solution). Leaking of phenolics into the floating solution could impose a serious constraint in interpretation.

2. Intact individual leaf study

Intact individual leaves held in water under 12 hr light/dark condition had very low rate of CO_2 evolution; 3 times lower than that found in the dark (Figure 2.5). This was due to fixation of CO_2 during photosynthesis in the 12 hr light/12 hr dark. In contrast, leaves in complete darkness were not photosynthesizing, and CO_2 production was due to respiration. Consumption of carbohydrates by respiration in the dark would be expected to deplete and finally use up all leaf carbohydrates. Respiration rate of leaves held in water in the dark declined significantly 8 days after harvest (Figure 2.5). This decline is possibly due to a lack of respiratory substrates. Leaves started to show the first signs of blackening 2 days after this respiratory decline (Figure 2.5) and the blackening rate increased dramatically as the respiration rate declined. Within 4 days, leaves were 100% black. A rise of respiration rate was observed after leaves became 100% black, probably due to fungal or bacterial infection as described by Coorts (1973). Leaves held in water in 12 hr light/dark did not show blackening.

Sucrose has been reported to provide respiratory substrates to cut flowers and therefore is included in most of preservatives (Halevy and Mayak, 1981). In complete darkness, intact individual leaves held in 5% (w/v) sucrose had significantly higher CO_2 evolution than leaves held in water 8 days after harvest, and remained higher than the control (Figure 2.5). Sucrose apparently provided the leaves with respiratory substrates in the dark and prevented leaf blackening.

Intact individual leaves held in water in 12 hr light/dark or held in 5% (w/v) sucrose in complete darkness did not turn black, and remained green and healthy. Leaves held in water in complete darkness began to show blackening 10 days after harvest, then the rate of blackening increased (Figure 2.5). Brown or black leaf exudates were observed on the blackened area. These leaf exudates contained 3 to 5 times more ion than washings from green leaves (Table 2.3), indicating the damage to cell membrane and leakage of cell substances. Metabolic sugars have been shown to protect membrane integrity (Goszczynska, *et al.*, 1990). Sucrose and glucose prevented intact individual leaf blackening in the dark, while sorbitol had no effect (Table 2.5). Protective effects of light on membrane integrity were not found in the literature, while in this study, light did prevent substance leakage and leaf blackening. The effect may be indirect by forming carbohydrates through photosynthesis. Reducing agents did not delay or prevent leaf blackening in the dark (Table 2.5), and the reason for this is not known.

Polyphenol oxidase (PPO) is responsible for the oxidation of phenolic compounds, a reaction required for forming brown or black pigments (Vaughn and

Duke, 1984). PPO has also been reported existing as a latent form embedded in chloroplast thylakoid membrane of green cells (Tobert, 1973; Vaughn and Duke, 1984). Upon senescence, PPO is released from the membrane, and becomes activated (Meyer and Biehl 1980, 1981; Vaughn and Duke, 1984). Extracting leaves with detergent such as SDS can also activates the enzyme (Sato and Hasegawa, 1976). In Protea leaves, very little soluble PPO activity was detected (Table 2.4) in green and healthy leaves (leaves held in water under 12 hr light/dark or leaves held in 5% sucrose under complete darkness). Detergent extraction increased PPO activity about 35 fold. This indicates that PPO in healthy protea leaves is latent, being probably activated following loss of membrane integrity. In partially blackened leaves (leaves held in water in the dark), soluble PPO activity increased, and the total PPO activity decreased (Table 2.4), indicating the release of PPO from the membrane to the cytosol. Membrane breakdown is probably responsible for the release of PPO. Chloroplast thylakoid membrane breakdown is the earliest event observed during leaf senescence (Thompson, 1988), and darkness is well known to induce leaf senescence (Bell, 1980; Goldthwaite and Laetscg 1967; Thimann *et al.*, 1977; Wittenbach, 1977). The effects of light and sucrose on preventing leaf blackening may be through preventing leaf senescence, protecting chloroplast membrane integrity, and maintaining PPO latency.

Using intact individual leaves detached from flower stems to study the blackening process was a useful model. The leaf is not very long (only about 13 cm) or wide (about 2 cm), and can easily be inserted into a 50 ml test tube making the

leaf easily handled, with the blackening process separated from flower head influences. The wounding to the leaf base is minimal compared to that with leaf discs. Results obtained with intact individual leaves therefore should more closely parallel the changes in leaves attached to flower stems than leaf discs. However, the influence of the flower head on cut flower leaf blackening process could be significant.

3. Flower stem study

Light compensation point of photosynthesis for most sun-plants is in the range of 10 to 20 μmol m^{-2} s^{-1} (Taiz and Zeiger, 1991). Protea leaves attached to flower stems after harvest are still able to carry out photosynthesis, with a light compensation point of about 10 μmol m^{-2} s^{-1} at 20 to 22 °C, 60 to 80% RH (Figure 2.6).

Light has a significant effect in improving vase life of chrysanthemum (Woltz, 1965; Woltz and Waters, 1967). Leaves in the light during storage are able to carry out photosynthesis thus prolonging cut flower's vase life. Light level significantly affected protea cut flower vase life (Figure 2.7). Light levels below compensation point reduced the cut flowers vase life more than 50%. Under dim light, close to light compensation point, flower bracts longevity was significantly longer than in the dark, although not as long as flowers held under high light level. Postharvest life of leaves attached to flower stems were significantly extended by light levels higher than the light compensation point (Figure 2.7).

Sucrose has been shown to improve postharvest life of many cut flowers (Halevy and Mayak, 1981). Under 12 hr light/dark condition, sucrose had little effect in preventing leaf blackening, and concentrations higher than 5% (w/v) were toxic to the leaves (Figure 2.10). However, under complete darkness, sucrose improved leaf vase life significantly up to a sucrose concentration of 5% (Figure 2.10). In the dark, 5% sucrose improved leaf vase life to about the same as leaves on a flower stem held under 12 hr light/dark.

Optimum concentrations of exogenous sugar in vase solutions for cut flowers to enhance longevity and quality varies with species, duration of treatment, and stage of floral development (Halevy and Mayak, 1981). Sucrose concentrations in the range of 0.5% to 1% are recommended for *P. neriifolia* to reduce the onset of leaf blackening (Newman *et al.*, 1990). McConchie *et al.* (1991) found that 0.5% sugar in the vase solution did not delay leaf blackening in the dark. In this study, sucrose concentrations up to 2.5% in the light significantly delayed the time to 50% leaf blackening while higher concentrations accelerated the condition (Figure 2.10). The reason of this difference is unknown. In darkness, 0.5% sucrose in vase solution was not sufficient to delay leaf blackening (Figure 2.10). In our present study, the optimum sucrose concentration to delay leaf blackening for flower stems held in the dark was determined to be 5% (Figure 2.10).

4. Summary

Darkness induces protea leaf disc senescence as measured by loss of photosynthetic activity and decline of protein and chlorophyll content. Intact individual leaves held in the dark, compared to that of in the 12 hr light/dark, had higher CO_2 evolution, higher soluble/total polyphenol oxidase ratio, more leakage of cell substances, and showed leaf blackening. Flower stems held in darkness or low light conditions had shorter vase life compared to stems held under bright light.

Light prevented leaf blackening. Under 12 hr light/dark, leaf discs bathing in water for 72 hr had higher photosynthetic activity (i.e. higher Fv/Fm ratio), higher protein and chlorophyll content than leaf discs in darkness. Intact individual leaves held in water in 12 hr light/dark had lower CO_2 evolution, lower soluble/total PPO ratio, and less leaf exudate, than leaves held in darkness; and showed no leaf blackening. The light compensation point for leaves still attached to flower stems was determined in this study as 10 μmol m^{-2} s^{-1}. Flower vase life was significantly extended when held under light levels higher than the light compensation point.

Sucrose prevented leaf blackening when proper concentrations were used. In the dark, sucrose at 5% (w/v) promoted intact individual leaves CO_2 evolution probably by providing respiratory substrates, prevented dissociation of PPO from the chloroplastic thylakoid membrane (represented by low soluble/total PPO ratio) and membrane leakage (low cell exudate), and significantly delayed both individual and flower stem leaf blackening. However, sucrose did not prevent the loss of photosynthetic activity in the leaf disc study. The effect of sucrose in preventing leaf

blackening thus can not be simply explained by provision of substrates for synthesizing reducing agents or producing a reduced environment to the leaf cell, since applying reducing agents to the bathing solutions or holding solutions also did not prevent the loss of photosynthetic activity of the leaf discs, and the blackening of the individual leaves.

In conclusion, lack of carbohydrates due to continuous dark respiration may lead to breakdown of membrane system. This breakdown of membrane system could cause the release and activation of PPO, a key enzyme in polyphenol oxidation, from the membrane. Damage of the membrane system was also shown by leakage of cell substances. Bright light or sucrose at a proper concentrations in the dark prevent the leaf blackening probably by providing carbohydrates (through photosynthesis if under light condition) for respiration and membrane protection. Intact individual leaves provide a useful model system for protea leaf blackening study.

This study confirms the hypothsis that darkness induces *Protea* leaf senescence thus leaf blackening, and light or sucrose prevents *Protea* leaf senescence and leaf blackening.

VII. SOURCE - SINK RELATIONSHIP AND LEAF BLACKENING

A. Introduction

Reproductive organs such as flowers, fruits, and seeds, are considered as nutrients sinks during growth (Ho, 1988). Mature leaves of a plant are the principle source of nutrients supply, especially carbohydrates supply to sink organs. Leaf senescence induced by growth and development of reproductive organs has been observed in many monocarpic crops (Leopold, 1961; Crafts-Brandner and Egli, 1987; Wittenbach, 1982; Nooden, 1984; Purohit, 1982; Ho and Below, 1989; Ho, *et al.* 1987). The hypothesis of this study is that *Protea* flower head is a major sink for carbohydrates. The flower head growth and nectar production affects the leaf carbohydrate status on the cut flower stem. Depletion of carbohydrates in leaves leads to leaf blackening. Providing flower stems with sugars or bright light, and flower head removal, may delay or prevent leaf blackening.

The objective of this study is to study the postharvest *Protea* flower growth (in terms of increased flower head diameter, flower head fresh and dry weight changes, and CO_2 evolution) and nectar production; to investigate the influence of flower head on leaf blackening rate, and to determine the relationship between the sink (flower head) demand and source (leaf) supply.

B. Materials and Methods

Plant Materials. *Protea* cut flowers at different stages of opening were harvested and grouped into five stages by degree of flower opening: Stage 1, Very

Tight Bud (bracts tightly closed); Stage 2, Not Quite Open (bracts began to loosen); Stage 3, Just Open (bracts started to open); Stage 4, Cylindrical (flower head opened with a cylindrical shape); Stage 5, Reflexed (bracts started to reflex outward). Stage 3 and 4 are usually considered as commercial harvesting stages.

Flower Growth and Development after Harvest. Flower head growth parameters (i.e. flower openness, flower head diameter, fresh and dry weight of flower head) at different stages were determined upon arrival from the field. Dry weight was determined after heating at 80°C for 48 hr. At each stage of opening, the flower head diameter (top and bottom of the flower) was determined.

Degree of flower opening and flower head diameter were determined continuously until the termination of the vase life. Initial results showed that stage 1 flowers (very tight flower buds) never opened after harvest, even when supplied with 5% (w/v) sucrose, or flower preservative. Therefore, stage 2 flowers were used to measure the continuous increase of flower head size. The diameter at the top of the flower head was used to represent the degree of flower opening.

Carbon dioxide rate. Flower head carbon dioxide evolution rate at different developmental stages was determined immediately after harvest and for stage 3 (commercial harvest stage) at 3 days, 6 days, and 9 days after harvest. Flower stems were held either in deionized water or in 5% (w/v) sucrose solution under 15 μmol m^{-2} s^{-1} fluorescent light, 20 to 22°C and 60% to 80% RH. The flower head was sealed in a 600 ml plastic jar for one hr, and a 1 ml sample of air taken and injected

into a LI-6251 Infra-red CO_2 analyzer (LI-Cor, Nebraska, U.S.A.). The results were expressed as CO_2 (ml kg^{-1} hr^{-1}).

Nectar Production Nectar production was determined after arrival from the field (initial) and seven days after harvest. The flower stems were held either in deionized water or 5% (w/v) sucrose solution. Nectar was collected by inserting the flower head upside down into a plastic cup, then spun with the International Centrifuge (Universal Model, International Equipment Co., Boston, Mass., U.S.A.) at 500 rpm for 1 minute. The nectar collected was measured with a graduate cylinder, and the Total Soluble Solids (TSS) determined with Abbe refractometer. Total sugar content in the nectar was determined by the method of Dubios *et al.* (1956), and reducing sugar content by the method of Nelson (1944).

Relative Proportion of flower head parts and the effects of flower head on leaf blackening. Flowers at stage 3 or 4 were harvested and trimmed to 46 centimeters long with 20 leaves. The stems were separated into three parts: flower head, leaves, and stem and weighed (fresh weight). The relative proportion of each part was calculated as the percentage of the whole shoot.

Removal of the flower head or girdling the stem just below the flower head by removing a 2 to 5 mm band of bark was used to alter leaf blackening rate. Stems were held in deionized water along with the control (intact flower stem) under 15 $\mu mol\ m^{-2}\ s^{-1}$, 20 to 22°C, and RH 60% to 80%. Leaf blackening rate was evaluated every other day and the days to 50% of leaf blackening was calculated.

Response of individual leaves to darkness and influence of flower head. One or two leaves on a flower stem were kept in the dark by wrapping the leaves with aluminum foil. Another one or two leaves's base were girdled, i.e. a band of the bark (about 2 mm wide) was removed from around the leaf base on the stem to cut the phloem, and then wrapped with aluminum foil. Leaves without aluminum foil wrap were used as controls. Leaves with base girdled but not wrapped with aluminum foil was used to check the effect of girdling. Flower stems were treated as follows: In deionized water, intact cut flower stems; flower stems with flower head removed; flower stems with flower head base girdled; and intact cut flower stems held in 2.5% (w/v) sucrose. Five flower stems were used for each treatment. Leaf blackening was evaluated every other day. The percentage of blackening on treated leaves 10 days after harvest was used to compare treatments.

Radioactivity labeling and flower distribution. A portion of a leaf (c.f. 0.5 cm^2) in the middle of the shoot was abraded, and 20 µl solution containing 74 kBq of uniformly labelled ^{14}C-sucrose (Cat.# NEC-100, Du Pont Company, NEN Research Products, Boston, MA. U.S.A.) was applied to the abraded area. Twenty-four hours later, the whole shoot was separated into bracts, florets, receptacle, nectar, and leaves. A small subsample from each fraction was placed in a scintillation vial containing 1 ml of ScintiGest (Fisher Scientific Company, New Jersey, U.S.A.) and digested 48 hours at 22°C. Toluene-scintillation liquid (10 ml) was added to the digested samples and radioactivity determined with a Beckman LS 1801 liquid scintillation counter (Beckman Instrument, CA, U.S.A.). The

radioactivity was expressed as disintegrations per minute (d.p.m.) gram^{-1} fresh weight. The radioactivity of individual parts was calculated as the percentage of the total radioactivity in the flower stem.

Effect of number of leaves on flower stem on leaf blackening and flower senescence. The ability of the leaf source to sustain the flower sink demand was hypothesized to be determined by the number of leaves on the flower stem; greater number of leaves greater ability to sustain flower head sink demand, therefore a slower rate of leaf blackening and flower head senescence. Leaves on flower stem at the commercial harvesting stage (flowers having cylindrical shape) were trimmed to 5, 10, 15, 20, and 25 leaves per flower stem. Stems were then held in deionized water. Flower head senescence and leaf blackening were evaluated as described in the General Materials and Methods section 7 days after leaf trimming.

Leaf net photosynthesis rate and leaf blackening. Net photosynthesis rate (NPR) of leaves attached to flower stems was determined with a LI-6000 Portable Photosynthesis System (Li-Cor, Nebraska, U.S.A.) as described in Chapter II, every other day after harvest. The hypothesis being that leaf photosynthesis activity is regulated by sink demand, and/or level of carbohydrates in the leaves. Flower stems were held in dim light (20 to 22 μmol m^{-2} s^{-1} PAR), at 20 to 22°C, 60 to 80% RH conditions.

Total sugar content in leaves attached to flower stems was determined 5 days after harvest by the method of Dubios *et al.* (1956) and described in Chapter II.

C. Results

1. Flower growth after harvest

Flower diameter at the top increased during development with a three fold increases (Figure 3.1), from 2.5 cm at stage 1 to 7.5 cm at stage 5. Flower diameter at the top was linearly related to stages of opening with a rate of increase of 1.3 cm per stage (r^2 = 0.99, $P<0.001$). The flower head diameter at the bottom also increased during development (Figure 3.1) at a rate of 0.21 cm per stage, 6 times slower than the top diameter. The relationship of the bottom diameter and stage of flower opening was linear (r^2 = 0.99, $P<0.001$).

Flower head top diameter increased slowly from 2.5 cm to 5 cm (4 to 6 days after harvest) when flower stems were held in deionized water (Figure 3.2), and completely stopped 8 days after harvest. The diameter of flowers held in 5% (w/v) sucrose continued to increase to about 8 cm 8 days after harvest, 38% higher than flowers held in deionized water (Figure 3.2), and reached 12 cm 15 days after harvest (Figure 3.2).

The fresh weight of the flower head increased only 0.1 gm per stage (Figure 3.3). Flower head dry weight did increased over the five stages, with a rate of increase of 1.2 gram per flower per stage. The increase in weight was linear (r^2 = 0.91, $P<0.001$).

Flower head fresh weight declined after harvest from about 80 gm to 55 gram (30% decrease) 12 days after harvest when stems were held in deionized water (Table 3.1). Flower head dry weight increased from 25 gram at the time of harvest

Figure 3.1. Flower head diameter changes at different opening stages. Flower head diameters were measured at bottom (o-----o) and top (●-----●) of the flower head. Regression analysis: Top diameter Y = 1.3 + 1.3X, r^2 = 0.99***; Bottom diameter Y = 4.4 + 0.21X, r^2 = 0.99***. Y = predicted diameter in cm., X = stage of flower opening. *** significance at 0.1% level, n = 10.

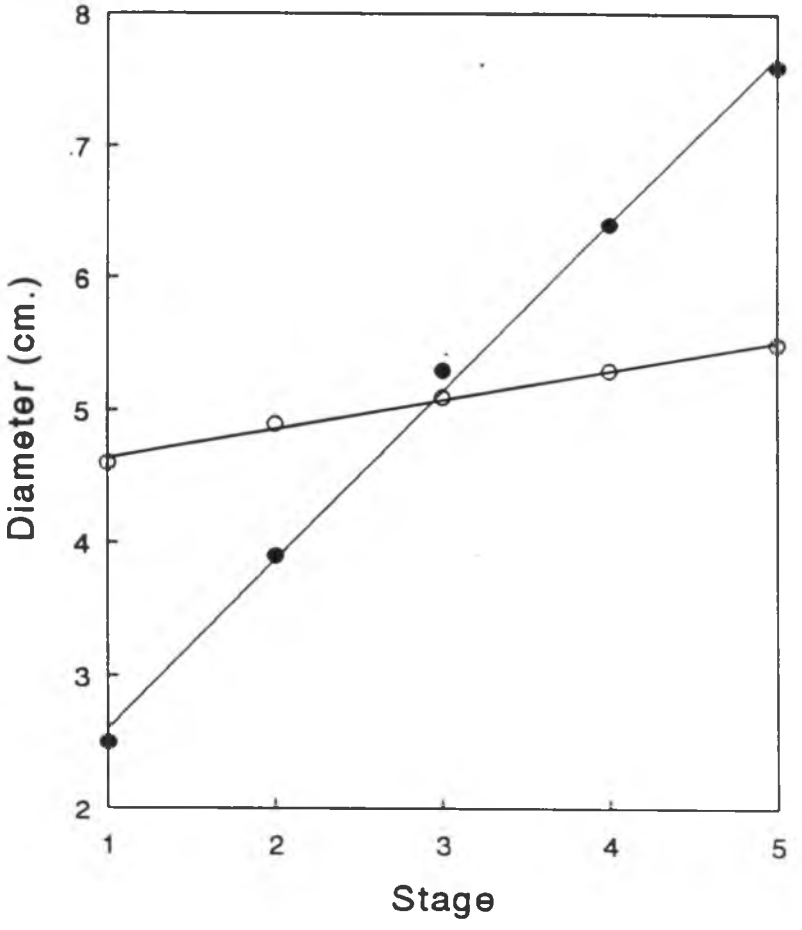

8
7
6
5
4
3
2
Diameter (cm.)
1
2
3
4
5
Stage

Figure 3.2. Top flower head diameter changes after harvest. Flower stems were either held in deionized water (o-----o) or 5% (w/v) sucrose (●-----●). In water: $Y = 2.5 + 0.69X - 0.034X^2$, $r^2 = 0.99^{***}$; in sucrose: $Y = 2.2 + 0.28X + 0.21X^2 - 0.027X^3 + 0.001X^4$, $r^2 = 0.99^{***}$. Y = predicted diameter in cm., X = days after harvest. *** significance at 0.1% level, n = 10.

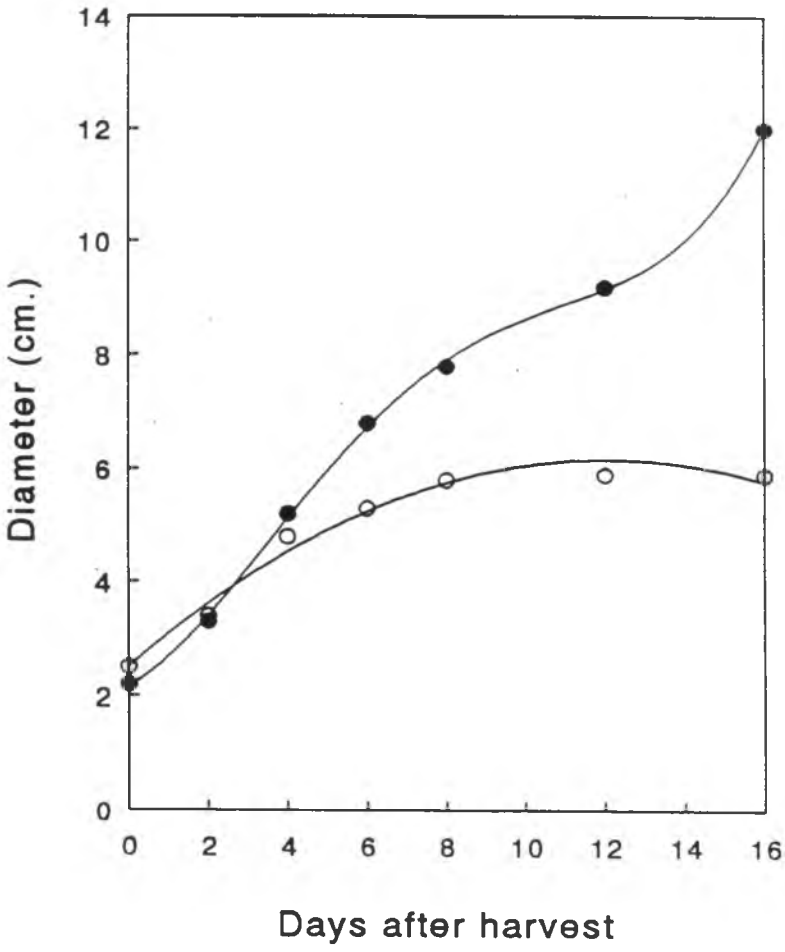

Diameter (cm.)
0
2
4
6
8
10
12
14
0
2
4
6
8
10
12
14
16
Days after harvest

Figure 3.3. Flower head fresh weight and dry weight changes at different stages of opening. o-----o, fresh weight, $Y = 71 + 0.1X$, $r^2 = 0.29^{ns}$; ●-----●, dry weight, $Y = 21 + 1.2X$, $r^2 = 0.91^{**}$. Y = predicted weight changes in gram, and X = stage of flower opening. ns, **, non-significant and significance at 1% level, respectively, n = 10.

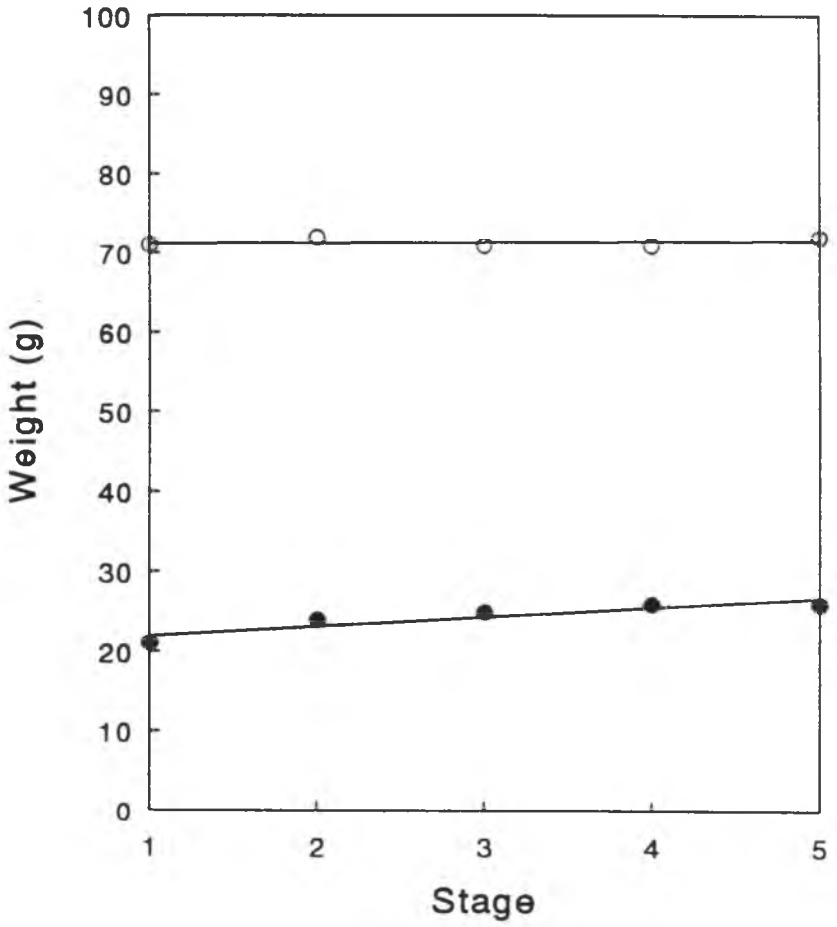

100
90
80
70
60
50
40
30
20
10
0
Weight (g)
1
2
3
4
5
Stage

Table 3.1. Flower head fresh and dry weight measured at the time of harvest (beginning) and 12 days after harvest (ending) while holding in either deionized water or 5% (w/v) sucrose solution.

Time after harvest	Fresh weight[z] g.	Dry weight g.
0	80.3 a	25 b
12 days		
DI water	55.0 b	27.1 a
5% (w/v) sucrose	54.2 b	28.0 a

[z]Data were analyzed by Duncan-Waller multiple range test, means with the same letter in the same column were not significantly different at 5% level, n = 8.

to 27 gram 12 days later. Holding flower stems in 5% (w/v) sucrose did not prevent flower head fresh weight loss, but increased dry weight significantly (Table 3.1).

2. Nectar production

At early stages of flower head development, nectar production was not detected (Table 3.2). At stage 4 when the flower head was open with a cylindrical shape, the first nectar production occurred, averaging about 3 ml per flower head. Nectar production rate increased after stage 4, and averaged about 10 ml at stage 5 when flowers frequently became sticky. Total soluble solids in the nectar increased from 15% at stage 4 to about 24% at stage 5 (Table 3.2). Florets of a stage 5 flower were fully mature, and noticeably shedding pollen. Seventy-five percent of the sugars found in the nectar were reducing sugars.

Little nectar was produced by flowers at all different stages of opening when the flowers were held in deionized water after harvest. Flowers at stage 4 produced 0.16 ml of nectar in seven days, with a total soluble solids of 21.7% (Table 3.3). When flower stems were held in 5% (w/v) sucrose solution for 7 days, production of nectar was detected at all opening stages but stage 1 (Table 3.3). Nectar production began at stage 2, 1.2 ml with a TSS of 12.5%. The highest production was found in flowers harvested at stage 3 and stage 4, with 3.3 ml and 3.9 ml per flower respectively. Although the amount of nectar production was not significantly different between these two stages, total soluble solids in the nectar of flowers harvest at stage 3 was significantly lower than those of stage 4 (Table 3.3). The

Table 3.2. Production and total soluble solid (TSS) of nectar produced at different stages of opening measured just after harvest from the field.

Stage of flower opening	Nectar[z]	
	Amount (ml per head)	Total soluble solid (%)
1	0 c	0 c
2	0 c	0 c
3	0 c	0 c
4	2.7 b	15 b
5	9.8 a	23.5 a

[z]Data were analyzed by Duncan-Waller multiple range test, means with the same letter in the same column were not significantly different at 5% level, n = 8.

Table 3.3. The amount of nectar production per flower and total soluble solid detected in nectar produced by flower heads at different stages seven days after harvest and after being held in either deionized water or 5% (w/v) sucrose solution.

Stage of Flower Opening	Nectar[z]			
	Held in water		Held in sucrose (5% w/v)	
	Amount[y] (ml)	TSS (%)	Amount (ml)	TSS (%)
1	ND[x]	ND	0.0 c	0.0 c
2	ND	ND	1.2 b	12.5 b
3	0.05 b	ND	3.3 a	16.3 b
4	0.16 a	21.7	3.9 a	21.6 a
5	0.05 b	ND	0.8 bc	23.7 a

[z]Data were analyzed by Duncan-Waller multiple range test, means with the same letter in the same column were not significantly different at 5% level. n = 8.

[y]Amount of nectar per flower head.

[x]None-detectable.

production of nectar declined significantly when flowers were harvested at stages 5 (0.8 ml), but the total soluble solids was higher at this stage (23.7%) than at any other stages.

3. Carbon dioxide evolution

Carbon dioxide evolution varied with stage of flower opening (Figure 3.4). The highest production was found at stage 1 (77 ml kg^{-1} hr^{-1}), declining significantly to 62 ml kg^{-1} hr^{-1} at stage 2, rising to 71 ml kg^{-1} hr^{-1} at stage 3, having a significantly low rate at stage 4 (59 ml kg^{-1} hr^{-1}) and 5 (56 ml kg^{-1} hr^{-1}). The high level of CO_2 production was found at stage 1 and 3 with no significant difference at the 5% level, and the CO_2 production at stages 2, 4, and 5 were significantly lower.

Carbon dioxide evolution by the flower head on the flower stems harvested at stage 3 is presented in Figure 3.5, with flower stems held in deionized water or 5% (w/v) sucrose solution. Carbon dioxide evolution from flower stem held in deionized water declined linearly with holding time. Initially (3 days after harvest), the CO_2 evolution was high (77 ml kg^{-1} hr^{-1}), then declined (35%) to about 50 ml kg^{-1} hr^{-1}, 6 days after harvest, and about 21 ml kg^{-1} hr^{-1} 9 days after harvest (Figure 3.5). There was a linear relationship between CO_2 evolution and days of holding:

CO_2 evolution = 106 - 28 days after harvest (r^2 = 0.99***)

Flower stems held continuously in 5% (w/v) sucrose after harvest had a significant quadratic relationship with days after harvest (r^2 = 0.99***). The evolution of CO_2 tended to decline with time (Figure 3.5). Overall, flowers held in

Figure 3.4. Carbon dioxide evolution of flower heads harvested at different stages of opening. Data were analyzed by Duncan Waller multiple range test. Bars with same letters were not significantly different at 5% level, n = 6.

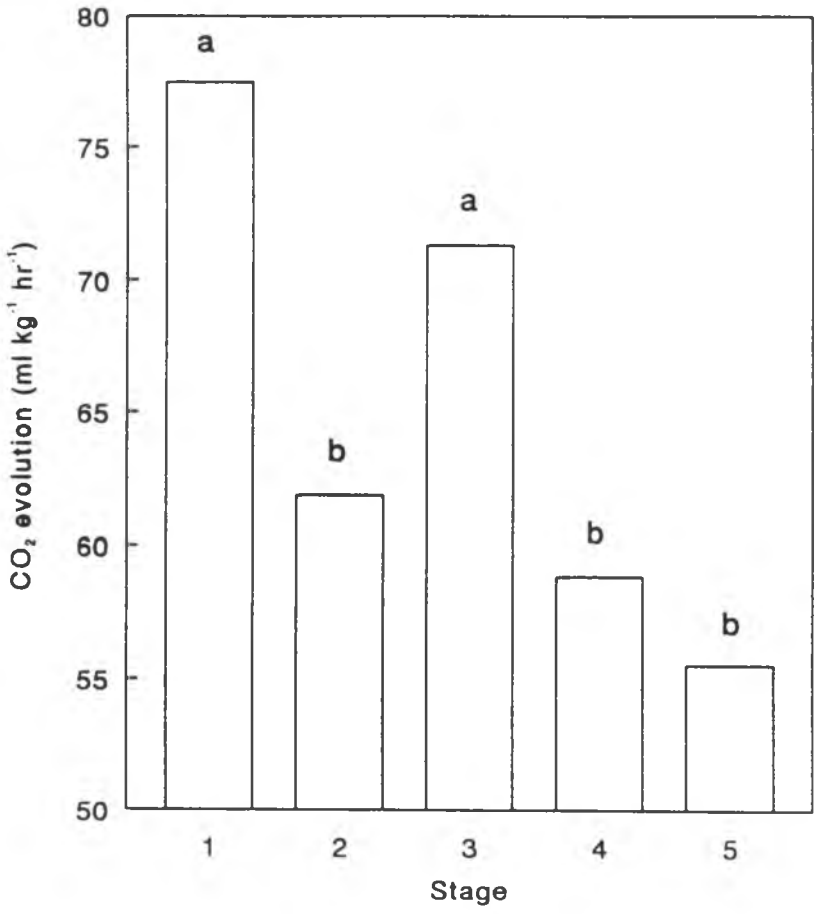
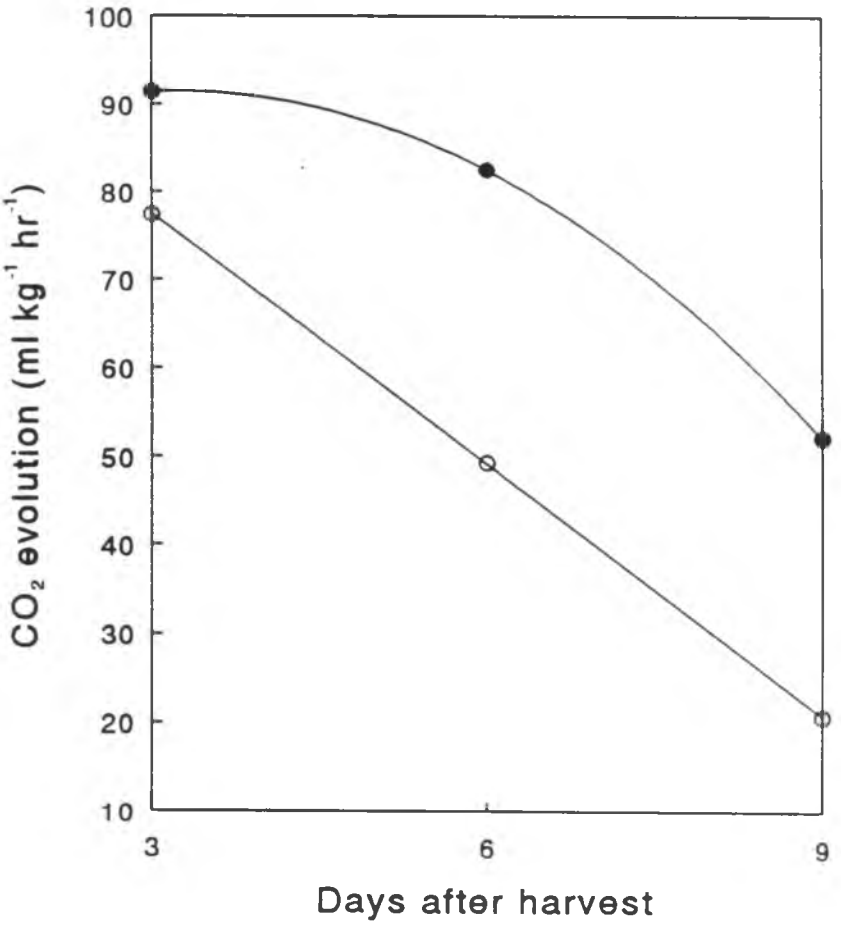

80
75
70
65
60
55
50
a
b
a
b
b
1
2
3
4
5
Stage
CO_2 evolution (ml kg^{-1} hr^{-1})

Figure 3.5. Carbon dioxide evolution from the flower head on the flower stems held either in deionized water (o-----o) or 5% (w/v) sucrose (●-----●) after harvest. In water: $Y = 106 - 28X$, $r^2 = 0.99^{***}$; in sucrose: $Y = 79 + 23X - 11X^2$, $r^2 = 0.99^{***}$. Y = predicted CO_2 evolution, and X = Days after harvest. *** significance at 0.1% level, n = 6.


100
90
80
70
60
50
40
30
20
10
3
6
9
CO_2 evolution (ml kg^{-1} hr^{-1})
Days after harvest

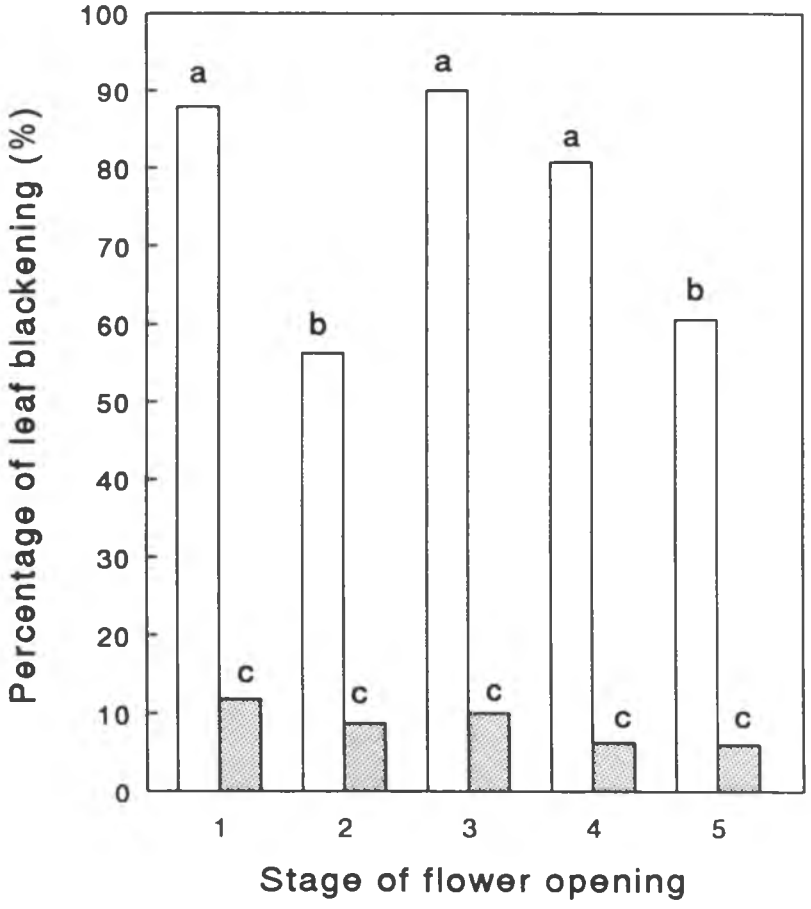
sucrose solution had higher CO_2 evolution rate than flowers held in deionized water. The CO_2 evolution was 91 ml kg^{-1} hr^{-1} 3 days after harvest, 15% higher than that of stems held in water and declined to about 83 ml kg^{-1} hr^{-1} 6 days after harvest, still 40% higher than the control (deionized water flower stem). When CO_2 evolution of control declined to 21 ml kg^{-1} hr^{-1} 9 days after harvest, the CO_2 evolution of sucrose treatment still remained 60% higher (52 ml kg^{-1} hr^{-1}), than the deionized water control.

CO_2 evolution = 79 + (23 × days from harvest) - (11 × days from harvest^2) (r^2 = 0.99, $P<0.001$).

4. Leaf blackening

Leaf blackening of flower stems harvested at different stages was determined 7 days after being held either in deionized water or 5% (w/v) sucrose solution. Percentage of leaf blackening was significantly higher in flowers held in deionized water than the sucrose treatment (Figure 3.6). When held in deionized water, the percentage of leaf blackening varied with flower opening stage (Figure 3.6), at stages 1, 3, and 4 had significantly higher percentage of leaf blackening (88%, 90%, and 80% respectively) than those at stages 2 and 5 (56% and 60% respectively). Sucrose 5% (w/v) significantly reduced leaf blackening at all stages, to less than 10% (Figure 3.6).

Figure 3.6. Percentage of leaf blackening on flower stems at different stages of opening, held either in deionized water (blank bars) or in 5% (w/v) sucrose (shaded bars) 7 days after harvest. Data were analyzed by Duncan Waller multiple range test. Bars with the same letters were not significantly different at 5% level, n = 8.


Percentage of leaf blackening (%)
100
90
80
70
60
50
40
30
20
10
0
a
c
b
c
a
c
a
c
b
c
1
2
3
4
5
Stage of flower opening

5. Source-Sink Relationship, Sucrose Supply and Leaf Blackening

Fresh Weight Distribution and Flower Head effect on Leaf Blackening. A flower stem with 20 leaves attached averaged about 120 grams (Table 3.4). The flower head was about 66% of the total fresh weight, stem 18%, and leaves 16% (Table 3.4).

Flower head removal, or girdling of flower head base significantly delayed leaf blackening (Table 3.5). The days to 50% of leaf blackening in the control was about 18 days, when the flower head was removed or base girdled the days to 50% of leaf blackening increased significantly to 24 days, a 30% of increase over the control. There was no significant difference between the effects of flower head removal and flower head base girdling (Table 3.5).

Leaf Blackening and Holding Conditions. Leaves attached to flower stems exposed to 12 hr light/dark did not show blackening 10 days after harvest, no matter whether their bases were girdled or not (Table 3.6). The same results were observed on stems with flower head removed, flower head base girdled, and intact flower stems held in sucrose solution (5% w/v). On intact flower stems, leaves wrapped with aluminum foil without base girdled had significantly higher percentage of blackening (60%) compared to leaves wrapped with aluminum foil (30%) but with leaf base girdled (Table 3.6). When the flower head influence was removed (either by flower head removal or flower head base girdling), leaves on the stem wrapped with aluminum foil but without leaf base girdled did not show blackening (Table 3.6), while leaves wrapped with aluminum foil and with leaf base girdled still had about

Table 3.4. Fresh weight distribution of different organs on a flower stem and their weight percentage of the whole flower stem.

Parts of flower stem	Fresh weight (g.)[z]	Percentage on flower stem
Whole shoot	116.75 ±16	
Flower head	77.19 ±11	66%
Leaves	18.12 ±5	16%
Stem	21.44 ±4	18%

[z]Data the means of 8 replicates, ±SD.

Table 3.5. Effect of flower head removal, flower head base girdling on delaying flower stem leaf blackening.

Treatments	Days to 50% leaf blackening[z]
Control	17.7 b
Flower head removed	23.6 a
Flower head base girdled	24.0 a

[z]Data were analyzed by Duncan-Waller multiple range test, means followed by the same letter were not significantly different at 5% level, n = 8.

Table 3.6. Percentage of leaf blackening on flower stems influenced by flower head and dark condition 10 days after harvest.

Treatments	% leaf blackening[z]
Intact flower	
Control	0
Leaf base girdled	0
Leaf in dark	60 a
Leaf girdled & in dark	30 b
Flower head removed	
Control	0
Leaf base girdled	0
Leaf in dark	0
Leaf girdled & in dark	25 b
Flower head girdled	
Control	0
Leaf base girdled	0
Leaf in dark	0
Leaf girdled & in dark	27 b
Intact flower in 5% sucrose	
Control	0
Leaf base girdled	0
Leaf in dark	0
Leaf girdled & in dark	0

[z]Data were analyzed by Duncan-Waller multiple range test, means followed by the same letter were not significantly different at 5% level, n = 9.

25% of blackening. The percentage of blackening on leaves wrapped with aluminum foil and with leaf base girdled were almost the same regardless of the influence of the flower head. Sucrose solution (5%, w/v) helped to prevent leaf blackening in all cases when leaves were wrapped with aluminum foil (Table 3.6).

Radioactivity Determination. Radioactivity distribution varied with stage of flower opening (Table 3.7). From the very tight bud to the just open stage, little nectar was detected and most of the ^{14}C radioactivity (90%) was found in the leaf fraction. The stems contained 5% of the radioactivity and declined during development. The flower head parts (bracts, florets, and receptacle) contained little radioactivity at all stages (Table 3.7). At stage 4, when the flower head was a cylindrical shape and began to produce nectar, the majority of the radioactivity (58%) was detected in the nectar (Table 3.7). Leaf radioactivity declined at this stage to 39%. When the flower head was fully reflexed (stage 5), the amount of radioactivity in whole shoot declined with nectar containing only 13% of the radioactivity. The radioactivity in nectar continued to decline after the stage 5, while the leaves appeared to increase to 97% of the radioactivity in whole shoot (Table 3.7).

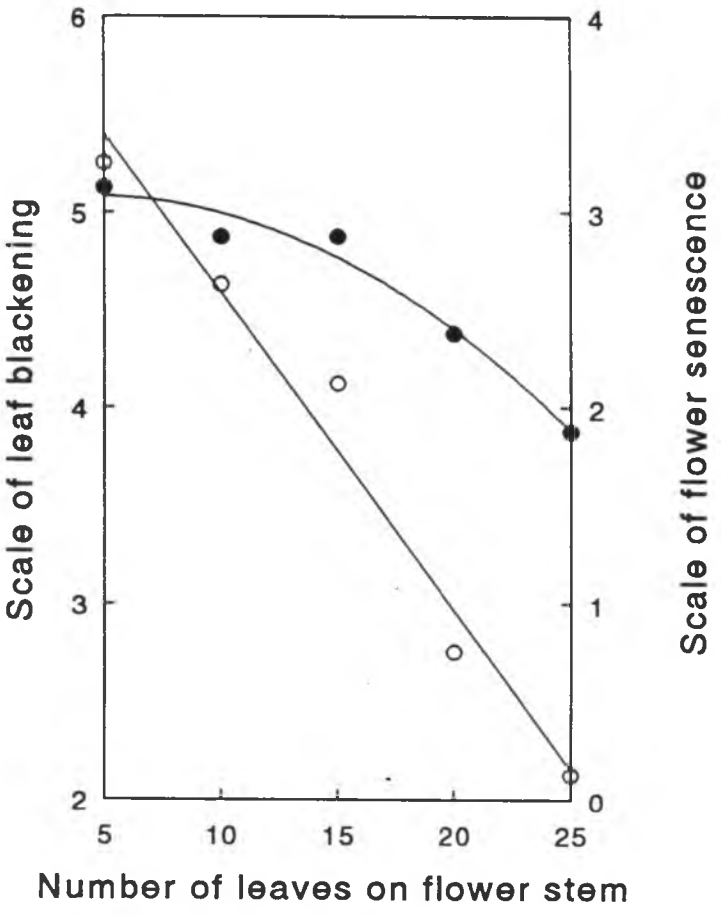
Number of Leaves on Flower Stem and the Rate of Leaf Blackening. The number of leaves on the flower stem significantly affected the rate of leaf blackening and flower senescence. The more leaves on the stem reduced the rate of leaf blackening and flower senescence (Figure 3.7). More than 60% of leaves became black (scales of leaf blackening higher than 4) when there were less than 15 leaves

Table 3.7. Distribution of ^{14}C in different parts of flower shoots at different stages of opening following 24 hours continuous exposure of ^{14}C-sucrose.

Section	Radioactivity (dmp g^{-1}) at six stages of flower opening[z]					
	V.T.B.[y] 1	N.Q.O. 2	Open 3	Cyl. 4	Refl. 5	> Refl.
Nectar	0	0	0	42164	3265	891
Stem	2498	1682	1997	620	812	125
L.L.[x]	19251	11374	11638	8585	7662	16098
M.L.	10540	9847	5588	9961	6377	15671
H.L.	15894	12430	10579	9628	6303	15126
Bracts	1276	215	384	631	185	40
Receptacle	416	572	246	138	297	74
Florets	908	1741	858	1010	534	191

[z]Data are the means from 3 replicates.
[y]Abbreviations: V.T.B., Very Tight Bud; N.Q.O., Not Quite Open; Cyl., Cylindrical; Refl., Reflex; >Refl., after Reflex.
[x]Abbreviations: L.L., the lowest leaf on the shoot; M.L., the middle leaf on the shoot; H.L., the highest leaf on the shoot. Radioactive material was applied to a middle leaf.

Figure 3.7. Effect of number of leaves on flower stem on leaf blackening (o-----o) and flower head senescence (●-----●) 7 days after harvest. For leaf blackening: $Y_1 = 6.2 - 0.16X$, $r^2 = 0.98^{***}$; bract senescence: $Y_2 = 3.1 + 0.014X - 0.0025X^2$, $r^2 = 0.99^{***}$. Y_1 = scale of leaf blackening, Y_2 = scale of flower senescence, and X = number of leaves on the flower stem. *** significance at 0.1% level, n = 6.


Scale of leaf blackening
Scale of flower senescence
Number of leaves on flower stem
6
5
4
3
2
4
3
2
1
0
5
10
15
20
25

on the stem, while less than 50% of leaves turned black (scale lower than 3) when more than 20 leaves still attached to the stem. The relationship between the number of leaves on flower stem and the scale of leaf blackening at 7 days after harvest was negatively related with a correlation coefficient (R^2 = 0.99). The relationship between the number of leaves on flower stem and the scale of flower senescence was a negative quadratic relationship (Figure 3.7).

6. Sink Demand and Source Activity

Sink Demand and Leaf Sugar Level Regulate Photosynthetic Activity. Net photosynthesis rate (NPR) of leaves attached to intact flower stems held in water, stems with flower head girdled held in water, or intact flower stems held in 5% (w/v) sucrose was compared. The NPR of leaves attached to the flower stem held in water was initially high (0.12 mg m^{-2} s^{-1}), 2 days after harvest (Figure 3.8), increasing to 0.19 mg m^{-2} s^{-1} 4 days after harvest, declining to the initial level 8 days after harvest, then rapidly declined to zero in the next 2 days (Figure 3.8). At the end of the experiment, leaves were still green and healthy, with only about 10% of leaf area (mostly leaf tip and edge) showing toxicity symptoms.

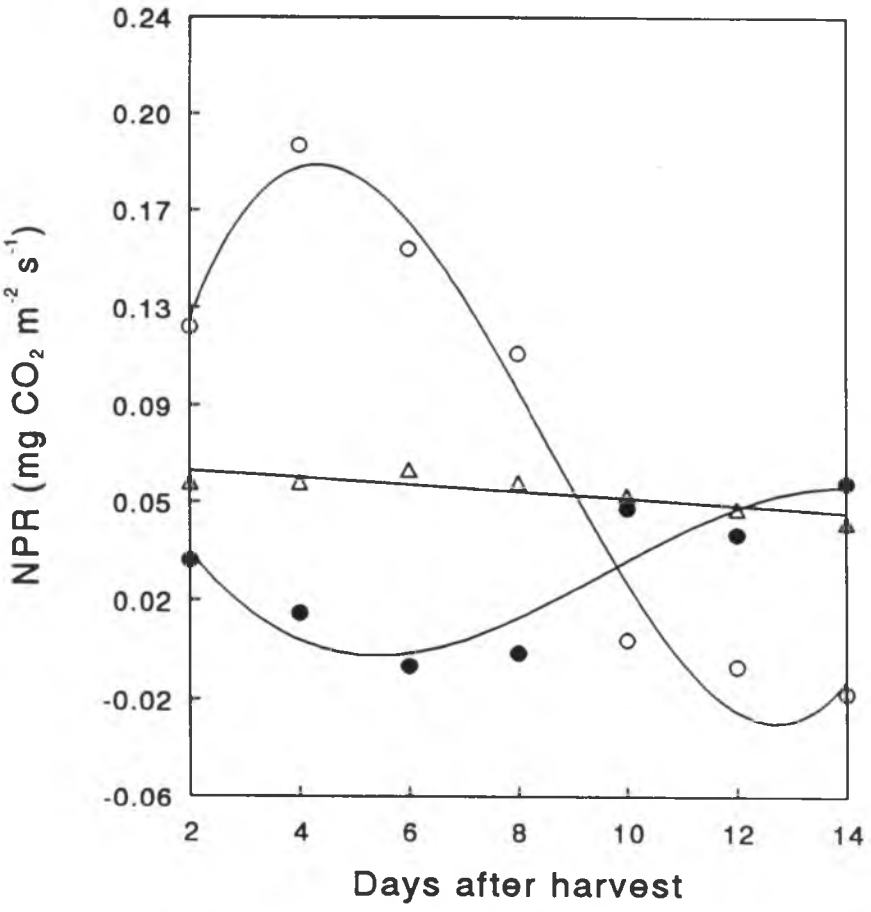
The net photosynthesis rate of leaves attached to stems with flower head removed remained constant throughout the experiment (Figure 3.8). The NPR was significantly lower than that of leaves attached to intact flower stems held in water through 2 to 8 days after harvest. The leaves still remained green and healthy at the end of the experiment.

Figure 3.8. Net photosynthesis rate (NPR) of leaves attached on intact flower stems held in deionized water (o-----o), or in 5% (w/v) sucrose solution (●-----●), or of leaves attached to stems with flower head removed and held in deionized water (▵-----▵).

Regression analysis:

Treatments	Equation[z]	r^2
Intact flower stem, water	$Y = 0.05+0.12X-0.019X^2+0.7\times10^{-3}X^3$	0.98***
Intact flower stem, sucrose	$Y = 0.11-0.047X+0.006X^2-0.2\times10^{-3}X^3$	0.90***
Flower head removed, water	$Y = 0.067 - 1.3 \times 10^{-3}X^3$	0.84**

[z]Y = leaf NPR, and X = days after harvest. **, *** significance at 1%, and 0.1% level respectively, n = 5.


0.24
0.20
0.17
0.13
0.09
0.05
0.02
-0.02
-0.06
NPR (mg CO_2 m^{-2} s^{-1})
2
4
6
8
10
12
14
Days after harvest

Leaves attached to intact flower stems held in water had the lowest sugar content in comparison to both those held in 5% (w/v) sucrose, or those with the flower head removed and held in water (Table 3.8). Sugar content of leaves on intact flower stems held in water was 50 mg. g^{-1} of fresh weight, 5 days after harvest, while leaves of flower stems treated with 5% sucrose had 117 mg. g^{-1}, double the sugar content. Leaves on stems with flower head removed and held in water had 77 mg. g^{-1} sugar content based on leaf fresh weight, 54% higher than those of leaves attached to intact flower stems. On a dry weight basis, sugar content in leaves attached to intact flower stems held in water was the lowest, with 118 mg. g^{-1}, 31% lower than in leaves attached to stems with the flower head removed (171 mg. g^{-1}) and 53% lower than leaves on intact flowers held in 5% (w/v) sucrose solution (Table 3.8).

D. Discussion

Reproductive organs, such as flower, fruit, or seed, are considered as strong nutrients sinks during growth (Ho, 1988). Sink strength, defined as the ability of a sink organ to import assimilates, is determined by sink size and sink activity (Daie, 1985). Protea cut flower is a strong sink with a large flower head consisting hundreds of florets. The flower head fresh weight was two thirds of the total flower stem weight (Table 3.4). From the flower bud to flower maturity stages, five stages of opening were distinguished according to the openness of the bracts of flower head. Although the flower head fresh weight did not change significantly during

Table 3.8. Total sugar content of leaves attached on flower stems held either in water or in 5% (w/v) sucrose under 22 μmol m^{-2} s^{-1} PAR 5 day after harvest.

Treatments	Sugar content[z] (mg. g^{-1})	
	Fresh weight	Dry weight
Water, intact flower	50.6 ±10.7	117.5 ±24.7
Water, flower removed	77.0 ±10.6	171.3 ±21.9
Sucrose, intact flower	117.3 ±20.3	248.7 ±46.1

[z]Data means of 5 replicate, ±SE.

flower opening, the dry weight did increase (Figure 3.2). Flower opening, measured as the top flower head diameter, increased significantly as the flower matured (Figure 3.1). Carbon dioxide evolution was highest at stage 1 and stage 3, when the flower was still at bud stage and had just began to open (Figure 3.3). This behavior is similar to many cut flowers in which the rate of respiration rises to a maximum as the flower start to open, followed by a gradual decline as the flower matures (Halevy and Mayak, 1979). *Protea neriifolia* is a member of the genus producing 5 to 6 ml of nectar per inflorescence with fructose and glucose as the dominant carbohydrates (Cowling and Mitchell, 1981). In the present study, nectar was found in flower heads that had a cylindrical shape and bract open at the top (Table 3.1). The volume and total soluble solids of the nectar increased as the flower matured (Table 3.1). The average volume of nectar collected was 9.8 ml per inflorescence at stage 5 (maximum 15 ml), with a total soluble solids of 23.5% (the highest 30%). The amount of sugar detected in such a flower head was about 2.4 gm on average (Table 3.1), and 5.25 gm on maximum. Seventy five percent of the total sugars detected in the nectar was reducing sugars (Table 3.1).

Carbohydrate is the principle substrate for cut flower respiration (Halevy and Mayak, 1979; 1981), but the mechanism of nectar production is not fully understood (Cowling and Mitchell, 1981). *Protea* flower stems held in deionized water after harvest had only the remaining leaves and stem as the carbohydrate source. The leaves were 34% of the total shoot fresh weight (Table 3.4). "Carbohydrate drain" by the flower head may cause the draining of carbohydrates from the remaining

reserves (leaves and stem) leading possibly to cell death. Flowers harvested at all developmental stages and held in deionized water had more than 50% leaf blackening 7 days after harvest (Figure 3.6). The rate of leaf blackening in Stages 1, 3, and 4 was higher than stages 2, and 5 (Figure 3.6). The reason for these difference in leaf blackening rate was unknown. It is possible that since respiration rate at stages 1 and 3 was higher than at stage 2 (Figure 3.3), and nectar production started at stage 4 and was declining at stage 5 (Figure 3.3 and Table 3.1), blackening rate would be lower at stage 2 and 5. Hence, stage 1, 3 and 4 of the flower head had the highest carbohydrate demand. Sucrose (5% w/v) in the vase solution may provide sufficient carbohydrate for the flower head and thus prevented leaf blackening (Figure 3.6). Leaves on flower stems held in sucrose were green and healthy, with only marginal browning possibly caused by the sucrose.

Flower bud opening in vase solution after harvest has been studied in cut chrysanthemums (Kofranek and Halevy, 1972), cut carnations (Paulin and Jamain, 1982), and *Dendrobium* 'Youppadeewam' flowers (Ketsa and Boonrote, 1990). Metabolizable sugars at different concentrations were found to be effective in promoting flower bud opening. *Protea* inflorescence expansion after harvest has been described by McConchie *et al.* (1991). In her study, inflorescence diameter increased from 5 cm at harvest to about 7 cm, 5 days after harvest when flower stems were held in deionized water, 0.5% sucrose did not promote inflorescence expansion. Our present study confirmed that inflorescence diameter did increase after harvest, from 2.5 cm at harvest to about 6 cm, 8 days after harvest when held in deionized water

(Figure 3.4). No further inflorescence expansion occurred after 8 days. Sucrose (5% w/v) significantly promoted flower opening, from 2.2 cm at harvest to about 8 cm, 8 days after harvest (Figure 3.4). The inflorescence held in sucrose continued to expand to about 12 cm, 15 days after harvest (Figure 3.4). Hence, the inflorescence held in water was not able to fully expand due apparently to a lack of carbohydrates. Sucrose at 0.5% (w/v) may not be sufficient to promote inflorescence expansion.

Carbon dioxide evolution after harvest has been studied in many cut flowers (Coorts, *et al.*, 1965; Marousky, 1969; Paull, *et al.*, 1985). In cut flowers of *Rosa Hybrida*, 'Velvet Times', respiration declined rapidly after harvest, and flower preservative containing 4% sucrose increased respiratory rate by 25-30% (Coorts, *et al.*, 1965). Similar results were obtained by Marousky (1969) for 'Better Times' roses. *Protea* flower florets respiration at different stages of opening and at different positions in the flower head correlates with temperature (Ferreira, 1986). The rate of respiration of *Protea* inflorescence florets after harvest shows a typical climacteric pattern (Ferreira, 1986). In our present study, CO_2 evolution was measured 3, 6, and 9 days after harvest and no climacteric respiration pattern was found. The respiration rate declined after harvest (Figure 3.5) with a rate of decline of 10 ml kg^{-1} hr^{-1} day^{-1}.

The inflorescence respiration rate decline after harvest was probably due to lack of respiratory substrate when flowers were held in water (Figure 3.5). Metabolic sugars are well known respiratory substrates for cut flowers (Halevy and Mayak, 1979, 1981). Sucrose has been described to be the major component of flower

preservatives contributing to increased respiration (Coorts, *et al.*, 1965). Sucrose (5% w/v) in the vase solution significantly increased CO_2 evolution by *Protea* inflorescences (Figure 3.5). The increase of respiration rate was double that of flowers held in water 9 days after harvest, 18%, and 67% higher 3 and 6 days after harvest. Sucrose obviously provided respiratory substrate for the *Protea* inflorescence after harvest.

Protea nectar production has been studied by Cowling and Mitchell (1981) on flowers still on the shrub. In the present study, inflorescences held in water did not produce significant amount of nectar during the 7 days of holding after harvest (Table 3.3). However, when held in a sucrose solution (5% w/v), significant amounts of nectar were found in the inflorescences harvested at all development stages (Table 3.3). Obviously, nectar production required carbohydrate supply, and sucrose in the vase solution can stimulate nectar production.

Mature leaves of a plant are the principle source of carbohydrates supply to sink organs (Ho, 1988). A conventional method to study source/sink relationship is to enclose the foliage in a $^{14}CO_2$ environment and then chase the ^{14}C radioactivity after a fixed period of photosynthesis. An extensive review of literature (Nooden, 1980a) indicates that ^{14}C-compounds derived from $^{14}CO_2$ through photosynthesis are distributed more to the reproductive organs than to the vegetative parts during the reproductive phase. In some cut flowers, ^{14}C compounds have been supplied via the vase solution. For example, in cut roses and carnations, movement of ^{14}C following a pulse of ^{14}C-sucrose was found to be selectively to leaves and stems (Sacalis and

Durkin, 1972), but not to flower heads during the pulse. However, during the distilled water chase, ^{14}C moved from leaves and stems into the flower heads and this movement diminished as the cut flowers aged. Sacalis and Durkin (1972) further established by girdling cut rose stems that assimilates are translocated from leaves and stems to flower heads via the phloem. Brink and de Swardt (1986) applied ^{14}C-sucrose to *Protea* flower vase solution for 18 hours, and found that ^{14}C accumulated a greater amount in the flower heads than in the leaves. In the present study, a leaf area was abraded, and U-^{14}C-sucrose was applied to the abraded area. The ^{14}C-radioactivity distribution to the different parts of the flower stems was determined after 24 hours. In young flower stem with flowers not fully open and no nectar production, most of the ^{14}C radioactivity was found in leaves (Table 3.7). When the inflorescence began to produce nectar, the radioactivity began to appear in the nectar (Table 3.7). However, when the flowers were more mature the radioactivity in nectar began to decrease. Nectar attracted more radioactivity than the rest of the stem when there was active nectar production. These data suggest that assimilates were translocated from leaves to the flower head, and especially to nectar production.

Sink demand regulating leaf photosynthetic activity has been observed in many source-sink relationship studies (Gucci, *et al.*, 1991; Foyer, 1988; Azcon-Bieto, 1983; Crafts-Brandner and Poneleit, 1987; Wittenbach, 1983). When the potential for photosynthesis in the leaf is not attenuated by a limitation in input, the photosynthetic system may be constrained by demand and become subject to

inhibition through low sink capacity (Foryer, 1988). *Protea* leaf photosynthetic activity after harvest was apparently regulated by the flower head and total sugar content of the leaf (Figure 3.8). When the flower head was removed, the sugar content in the leaf increased and photosynthetic activity decreased (Figure 3.8). The lowest photosynthetic activity was found in leaves having the highest total sugar content when flower stems were held in sucrose (5% w/v) solution.

Leaf senescence induced by growth and development of reproductive organs has been observed in many monocarpic crops (Leopold, 1961; Crafts-Brandner and Egli, 1987; Wittenbach, 1982; Nooden, 1984; Purohit, 1982; Ho and Below, 1989; Ho, *et al.* 1987). Sink demand for carbohydrates after harvest may deplete carbohydrates in the leaves, leading to a disruption of compartmentation and leaf blackening (McConchie, *et al.*, 1991). Sink removal has been found to delay whole plant senescence in many monocapic plants (Leopold, 1961; Ho, *et al.* 1987). *Protea* flower head removal, or girdling flower head base has been found to delay leaf blackening (Reid, *et al.*, 1989). Our results (Table 3.5) confirm the results of Reid *et al.* (1989) and McConchie *et al.* (1991), indicating that *Protea* flower head promotes leaf blackening by draining carbohydrate from the leaves.

Leaf blackening on flower stems after harvest was not only influenced by flower head, but also affected by darkness (Table 3.6). Leaves with base girdled and kept in the dark blackened at the same rate regardless of the flower head influence (with or without flower head). Depletion of carbohydrate due to leaf respiration may lead to this blackening. Our previous study (Chapter 2) also showed that individual

leaves kept in the dark had higher respiration rates and blackened, while leaves under 12 hr light/dark had lower respiration rates and did not blacken (Figure 2.7). Leaves on flower stems kept in the dark but without base girdled had higher percentage of leaf blackening, twice as high as those with leaf base girdled. It was hypothesized that leaves without a girdled base and kept in the dark were depleted of carbohydrates more quickly than those with base girdled, since the former were also influenced by flower head demand. Leaves with sucrose supplied did not blacken at all. Therefore, carbohydrates were the main factors affecting *Protea* flower leaf blackening.

The number of leaves on the flower stems affected flower longevity and the degree of leaf blackening, with more leaves on flower stem having longer flower vase life and less leaf blackening (Figure 3.7), further suggesting the importance of the relationship between leaf carbohydrate source supply and flower head sink demand. Flower stems with a stronger carbohydrate source supply (more leaves on stem) had a longer vase life, while reducing sink demand by girdling the flower head base reduced the degree of leaf blackening. A greater carbohydrate supply can also be achieved by holding flower stems under bright light condition, or adding sugars to the vase solution.

VIII. COMPARISON OF LEAF SUSCEPTIBILITY TO ENZYMATIC BLACKENING IN *Protea neriifolia* R. Br. AND *Leucospermum* 'RACHEL'

A. Introduction

As mentioned in the Literature Review, most plant tissue browning or blackening are initiated by enzymatic oxidation of phenolic compounds (Labuza and Schmidl, 1986; Nimiki, 1988), and one of the enzyme responsible for this enzymatic browning or blackening is polyphenol oxidase (PPO). Degree of browning in many fruits during postharvest handling has been found to positively correlate with PPO activity (Kahn, 1975; Jayaraman *et al.*, 1982; Sapis *et al.*, 1983). But in most plants, the potential of enzymatic browning or blackening is determined by enzyme activity, substrate concentration, and other endogenous substances such as ascorbic acid, pH and nature enzyme inhibitors (Ben-Shalom *et al.*, 1977; Jayaraman and Ramanuja, 1987; Omuaru *et al.*, 1990).

The hypothesis of this study is that postharvest *Protea* leaf blackening is highly related to polyphenol oxidase activity. Species or genera that do not show leaf blackening may lack PPO activity or have inhibitors of PPO activity.

Leucospermum, another genus in the family of Proteaceae, is also an important cut flower in *Protea* cut flower industry around the world as well as in Hawaii (Kepler, 1988). Leaf blackening of *Leucospermum* species is not reported in the literature. Therefore, the objectives of this chapter is to compare leaf susceptibility to blackening in *Protea* and *Leucospermum*, to study their leaf biochemical

characteristics, and to investigate the relationship of these biochemical characteristics and the potential of leaf blackening.

B. Materials and Methods

Plant materials. Healthy, mature green leaves were detached from flower stems of *Leucospermum* 'Rachel' and *Protea neriifolia*, and used for enzymatic browning or blackening determination. *L.* 'Rachel' was a selected seedling of a cross using *L. lineare* x *L. vestitum* as the female parent and pollen from *L. glabrum* as the male parent (Ito *et al.*, 1992). For biochemical analysis, leaves were washed, blotted dry and ground with a pestle and mortar into powder in the presence of liquid nitrogen (midvein not included). The leaf powder was then immediately used for analysis or stored in -21°C for further use.

1. Leaf's susceptibility to enzymatic browning or blackening

Leaf susceptibility to blackening determination. A small area (about 5 mm in diameter) of leaf surface of the two genera was abraded, and the wound was exposed to the air for a few minutes for oxidation to occur. Leaf susceptibility to blackening was determined by observing the color change of the wounded area.

Leaf disc study. The method for leaf disc study was the same as described in Chapter II.

Whole flower stems. Flower stems of *Leucospermum* and *Protea* were held in deionized water and placed in 12 hr dim light (7-9 μmol m^{-2} s^{-1})/dark condition at

20 to 22°C and 60% to 80% RH. Leaf condition was evaluated every other day as described in previous chapters.

2. Physical and biochemical characteristics

Dry weight percentage. One gram of fresh leaf tissue from the two genera were oven dried at 80°C for 48 hr, and the dried material weighed. Dry weight percentage was calculated as the dry weight over the fresh weight times one hundred.

Polyphenol oxidase (PPO) extraction and assay. PPO extraction and assay for both genera were carried out in the same way as described in chapter II. To test the optimum substrate concentrations for PPO reaction, 1 to 100 mM of catechol was used. When the effect of *Leucospermum* leaf extract on *Protea* PPO activity was studied, 10 µl or 15 µl of *Leucospermum* leaf extract (protein content: 11 µg and 16.5 µg, respectively) was added to the reaction solution prior to *Protea* leaf extract addition (protein content: 44.7 µg), and PPO activity determined as described previously.

Total protein assay. The partially purified crude extract (the filtrate) was precipitated in cold acetone overnight (1:3). After at least 3 washes with cold acetone, the precipitate was resuspended in the Tris-HCl buffer (125 mM Tris-HCl only), and protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Ascorbic acid content. Ascorbic acid in the leaf tissue was determined by dye reduction assay of Loeffler and Ponting (1942).

Phenolic content. Water soluble phenolic content was determined by extracting 1 g of ground leaf powder with water. Phenolic content was measured by the Folin Phenol reagent method (Singleton and Rossi, 1965), with catechol as the standard. Tannic acid was determined by the method of Schultz *et al.* (1981). Total phenolics was determined by extracting the leaf powder with 50% of methanol, and measured as described by Schultz *et al.* (1981).

pH determination. Ground leaf powder (2 gm) was homogenized in 10 ml water, and pH was determined with standard digital pH meter.

3. Enzymatic blackening in leaf extracts

A sample of leaf powder (2 gm) was mixed with 10 ml deionized water in an ice bath. After centrifugation at 10,000 g for 10 min at 0-4°C, the supernatant was transferred to a test tube, and allowed to stand at room temperature for 48 hrs. To determine whether *Leucospermum* has an inhibitory effect on PPO activity, the supernatants of the two genera was mixed in a 1:1 or 1:9 ratios, using a mixture of *P. neriifolia* extract with water (1:1) as the control. The browness of the supernatants and the mixtures were determined visually and absorbance measured at 490 nm. Absorption spectrum of the supernatants was also compared.

C. Results

Leaf susceptibility to enzymatic browning. The wounded area of *P. neriifolia* leaves became brown and the color intensified to black in 2 to 5 minutes of exposure to the air, while the wounded area on the *Leucospermum* leaf remained green (Figure 4.1). *P. neriifolia* leaf discs bathing in water in the dark began to show edge browning 24 hours after incubation. The whole leaf disc became brown 48 hours after incubation (Figure 4.2). *Leucospermum* leaf discs remained green, although water soaked symptoms were observed on the leaf disc (Figure 4.2).

The visible spectrum of leaf disc bathing solutions from both genera were determined after 48 hours of incubation. The absorbance (400 to 800 nm) showed significant differences (Figure 4.3). Bathing solution of *Leucospermum* leaf discs had very low absorbance in the visible spectrum, while absorption peaks were found around 400 nm and 500 nm in *Protea* leaf disc bathing solution (Figure 4.3).

The bathing solution of *Protea* became reddish brown 24 hours after incubation. After 48 hr, the browness of the solution intensified. Seventy-two hours after incubation, the bathing solution of *Protea* leaf discs had become dark-brown, and had the highest (ca. 0.45) absorbance at 490 nm (Figure 4.4). The increase in absorbance at 490 nm of *Protea* bathing solution was linear with time (5.9×10^{-3} absorbance unit per hour).

The bathing solution of *Leucospermum* leaf discs remained clear, and had very low absorbance (<0.02) at 490 nm (Figure 4.4). The absorbance of Protea leaf discs bathing solution at 490 nm was more than 20 times higher than that of

Figure 4.1. Comparison of leaf susceptibility to blackening after injury in *Leucospermum* and *Protea*. Left: *Leucospermum*; Right: *Protea*.

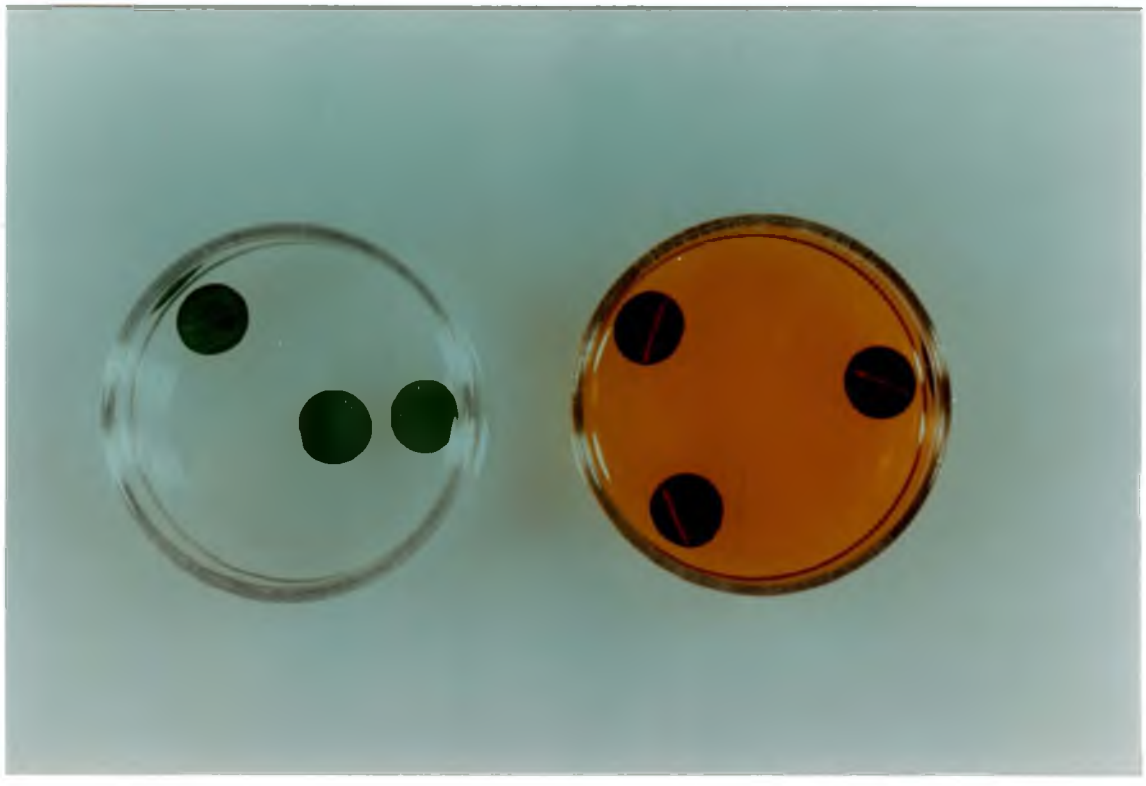


Figure 4.2. Comparison of leaf disc susceptibility to blackening after incubation of disc in water in the dark. Left: *Leucospermum*; Right: *Protea*.

Figure 4.3. Spectrum of *Protea* and *Leucospermum* leaf disc bathing solution after 48 hr incubation in deioninzed water.

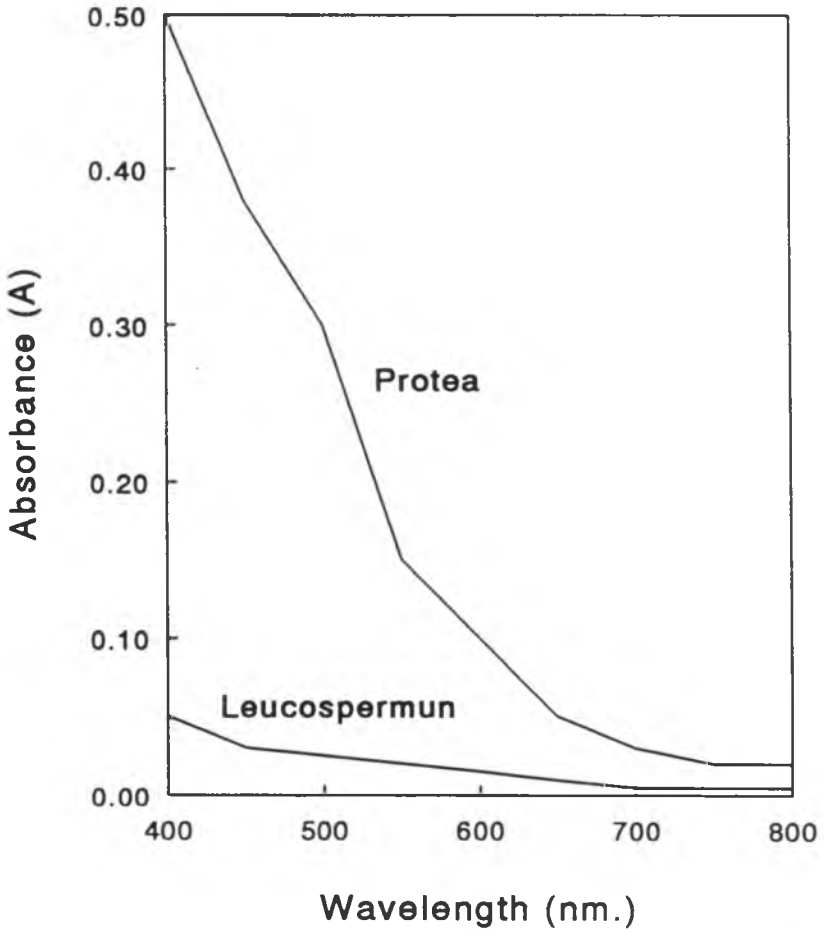
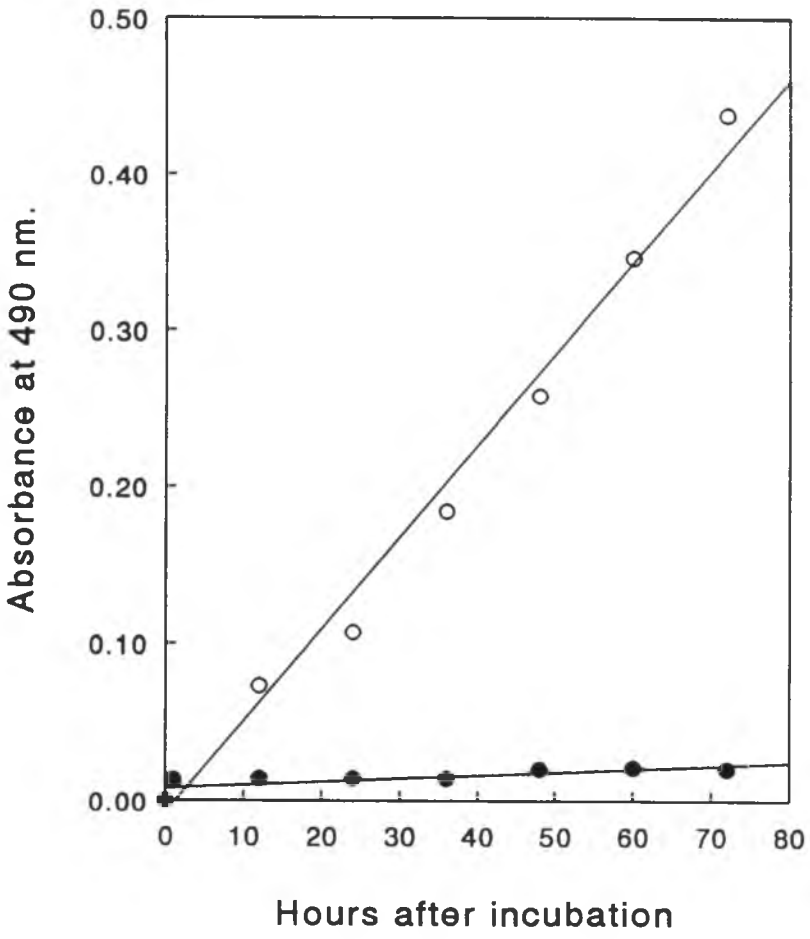

0.50
0.40
0.30
0.20
0.10
0.00
Absorbance (A)
Protea
Leucospermun
400
500
600
700
800
Wavelength (nm.)

Figure 4.4. Absorbance of *Leucospermum* (●-----●) and *Protea* (o-----o) leaf disc bathing solution at 490 nm after incubation. For *Leucospermum*: $Y = 7.9 \times 10^{-3} + 0.2 \times 10^{-3}X$, $r^2 = 0.82^{**}$; *Protea*: $Y = -7.8 \times 10^{-3} + 5.9 \times 10^{-3}X$, $r^2 = 0.99^{***}$. Y = absorbance at 490 nm, and X = incubation time. *** significance at 0.1% level, n = 6.


0.50
0.40
0.30
0.20
0.10
0.00
Absorbance at 490 nm.
0
10
20
30
40
50
60
70
80
Hours after incubation

Leucospermum 72 hours after incubation (Figure 4.4). The rate of increase in absorbance at 490 nm was slow for *Leucospermum*, (0.2×10^{-3}) increase in absorbance unit per hour.

Characterization of two genera. The dry weight percentage of *Protea* leaf tissue was 48%, while that of *Leucospermum* leaf tissue was only 30%, 18% lower than *Protea*'s (Table 4.1). Total protein in *Leucospermum* leaf was 5.46 mg g^{-1} fresh weight, only one-forth of *Protea*'s (22.35 mg g^{-1} fresh weight). Polyphenol oxidase activity was not detected in leaves of *Leucospermum*, while in *Protea* leaves, 14 units of PPO activity was detected based on the same amount of protein (20 μg). There was no significant difference in ascorbic acid content in leaves between the two genera; both had ca. 3.0 mg g^{-1} fresh weight (Table 4.1). Water soluble phenolics content between the two genera was not significantly different based on leaf fresh weight, and was 18 mg g^{-1} for *Leucospermum* and 24 mg g^{-1} for *Protea* (Table 4.1). However, there was significant difference in tannic acid and total phenolics based on leaf fresh weight (Table 4.1). The leaf tannic acid in *Protea* was 121 mg g^{-1} fresh weight (Table 4.1), two times higher than that in leaves of *Leucospermum* (61 mg g^{-1} fresh weight). Total phenolics in *Leucospermum* leaves was 108 mg g^{-1} fresh weight, and 393 mg g^{-1} of fresh weight in *Protea* leaves (Table 4.1), almost 4 times higher than in *Leucospermum* leaves. *Protea* leaf tissue had significantly higher pH value (5.2) than *Leucospermum* (pH = 4.4) (Table 4.1).

Blackening of leaf extract. The leaf extracts were checked immediately, 3 hr, 6 hr, 12 hr, 24 hr, and 48 hr after extraction. No blackening was observed in *Leucospermum*

Table 4.1. Comparison of some characteristics of *Protea neriifolia* and *Leucospermum* 'Rachel' leaf tissues.

Characteristics	Leucospermum[z]	Protea
Dry weight %	30 ±2	48 ±2
Total protein (mg g^{-1} fresh wt.)	5.46 ±0.7	22.35 ±2.2
Total PPO activity (unit/ml)	N.D[y]	14 ±1.2
Ascorbic acid (mg/g. fresh wt.)	3.0 ±0.1	2.9 ±0.2
Water soluble phenolics (mg/g.fresh wt)	18 ±2	24 ±4
Tannic acid (mg g^{-1} fresh wt.)	61 ±11	121 ±21
Total phenolics (mg/g. fresh wt.)	108 ±18	393 ±42
pH	4.4 ±0.1	5.2 ±0.1

[z]Means ±SE, n = 3.

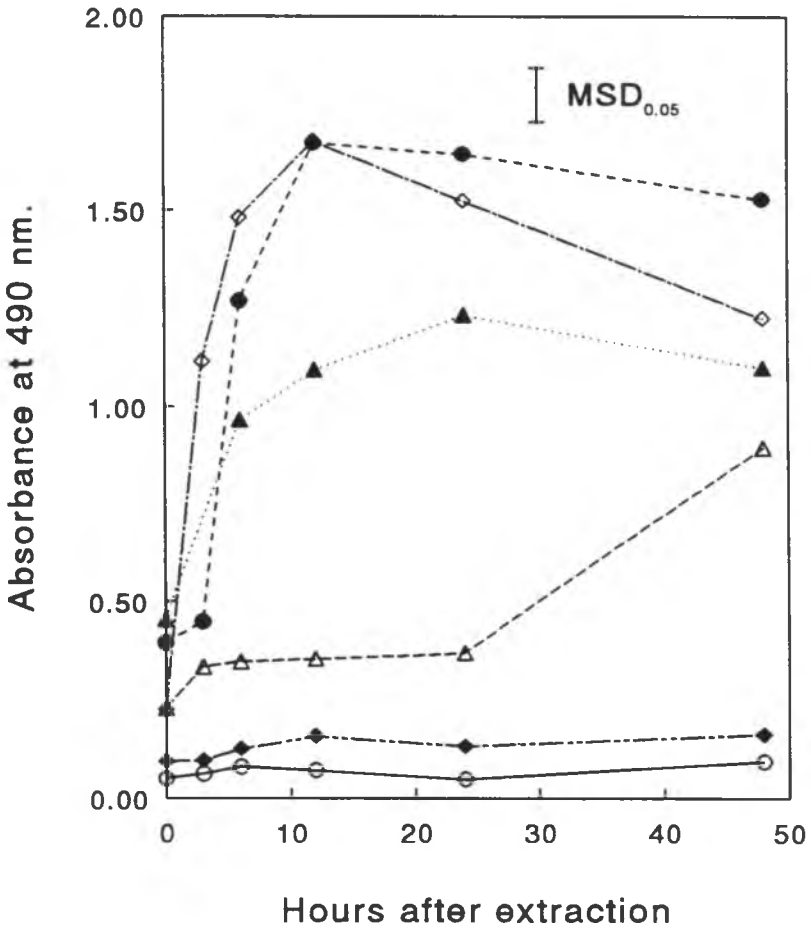
[y]Not detectable.

leaf extract or the mixture of *P. neriifolia* and *Leucospermum* leaf extract at 1:9 ratio following 48 hours incubation. Little blackening occurred during extraction or immediately after extraction of *P. neriifolia* leaf extract, but blackening was observed after standing for 6 hr. The blackening started to occur in the upper layer of the extract where the extract was exposed to the air, then moved down to the bottom of the tube. The degree of blackening intensified with time. Twelve hours later, *P. neriifolia* leaf extract was completely black. The observation was similar to the mixture of *P.neriifolia* and *Leucospermum* leaf extract at a 9:1 ratio.

Visual observations were confirmed by measuring leaf extract absorbance at 490 nm. The absorbance of *Leucospermum* leaf extract, or the mixture of *P. neriifolia* and *Leucospermum* leaf extract at 1:9 ratio remained low and constant throughout the period of incubation (Figure 4.5), while the *P. neriifolia* leaf extract had a three-fold increase in absorbance in the first 6 hr (Figure 4.5), and reached a maximum 12 hr after extraction with no subsequent absorbance increase. The same result was obtained for mixtures of *P. neriifolia* and *Leucospermum* leaf extract at 9:1 ratio (Figure 4.5).

The mixture of *P. neriifolia* and *Leucospermum* leaf extract at a ratio of 1:1 did not blacken in the first 24 hr and the absorbance at 490 nm was low compared to the mixture of *P. neriifolia* with water (Figure 4.5). Blackening of the former first appeared 48 hr later when the absorbance at 490 nm increased 3 fold (Figure 4.5). However, the intensity of the blackness never reached the same degree as the *P. neriifolia* leaf extract alone. The mixture of *P.neriifolia* with water was less black and

Figure 4.5. Absorbance of *Leucospermum* leaf extract (o-----o), *Protea* leaf extract (●----●), mixture of *Protea* and *Leucospermum* leaf extract at 9:1 ratio (◇-----◇), 1:9 ratio (◆----◆), 1:1 ratio (▵-----▵), and mixture of *Protea* with water 1:1 (▲----▲) at 490 nm after incubation. Data were analyzed by Duncan-Waller multiple range test, $MSD_{0.05}$ = mean significant difference ($P<0.05$, $n = 6$).


2.00
1.50
1.00
0.50
0.00
Absorbance at 490 nm.
MSD0.05
0
10
20
30
40
50
Hours after extraction

the absorbance at 490 nm was lower than either pure *P. neriifolia* leaf extract or mixture of *P.neriifolia* and *Leucospermum* leaf extract at 9:1 ratio, but was more black and had significantly higher absorbance at 490 nm than mixture of *P.neriifolia* and *Leucospermum* leaf extract at 1:1 ratio (Figure 4.5).

PPO activity. PPO activity was not detected in *Leucospermum* leaf extract or mixture of *P.neriifolia* and *Leucospermum* leaf extract at a 9:1 ratio (Figure 4.6). For *P. neriifolia* leaf extract, there was no detectable activity immediately after extraction, but maximum activity was detected 6 hours later (Figure 4.6) and subsequently activity declined. Low activity was detected after 24 hr of standing. Polyphenol oxidase activity in mixture of *P. neriifolia* and *Leucospermum* leaf extract at 9:1 ratio was significantly lower than pure *P. neriifolia* leaf extract.

Polyphenol oxidase activity in the mixture of *P. neriifolia* and *Leucospermum* leaf extract at 1:1 ratio was not detected until 12 hours after extraction (Figure 4.6). But the activity was very low compared to the *P. neriifolia* leaf extract. Polyphenol oxidase activity in the mixture of *P. neriifolia* and water was initially very high, and subsequently declined (Figure 4.6).

Ascorbic acid content. Initially, *Leucospermum* and *P. neriifolia* had the similar ascorbic acid content on a leaf fresh weight basis (Figure 4.7). Ascorbic acid content in *Leucospermum* leaf extract or a mixture of *P. neriifolia* and *Leucospermum* leaf extract at 1:9 ratio did not decrease significantly during 48 hr incubation (Figure 4.7). In *P. neriifolia* leaf extract, or mixture of *P. neriifolia* and *Leucospermum* leaf

Figure 4.6. Polyphenol oxidase activity detected in leaf extracts of *Protea* (o----o), a mixture of *Protea* leaf extract with *Leucospermum* at 9:1 ratio (▲-----▲), at 1:9 ratio (◆----◆), at 1:1 ratio (◇-----◇), and mixture of *Protea* leaf extract with water 1:1 (△-----△). Data were analyzed by Duncan-Waller multiple range test, $MSD_{0.05}$ = mean significant difference ($P<0.05$, $n = 6$).

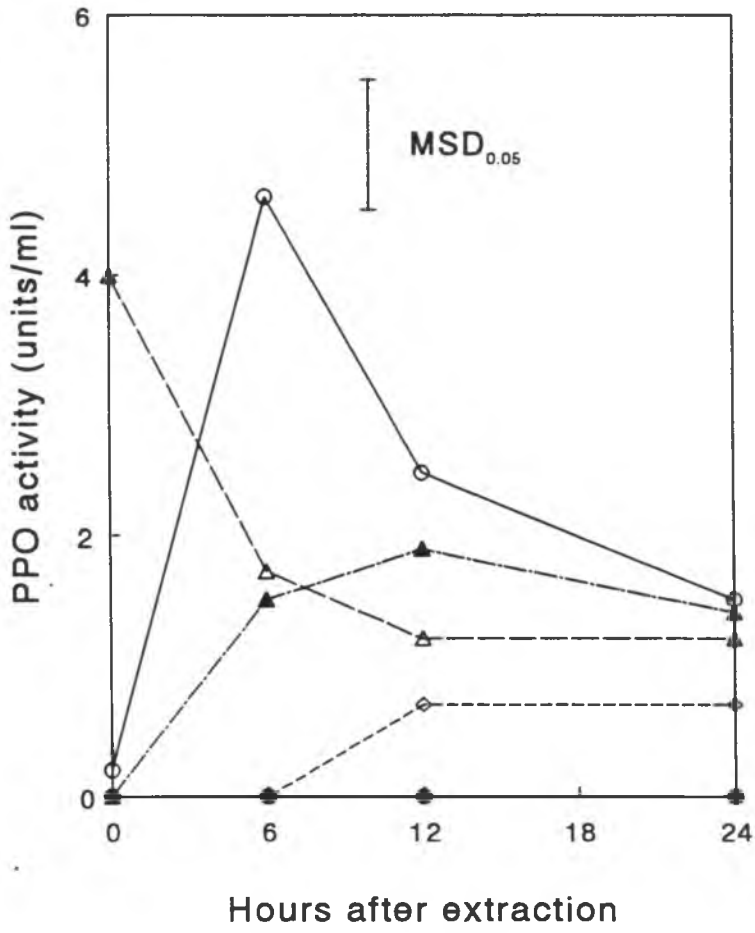
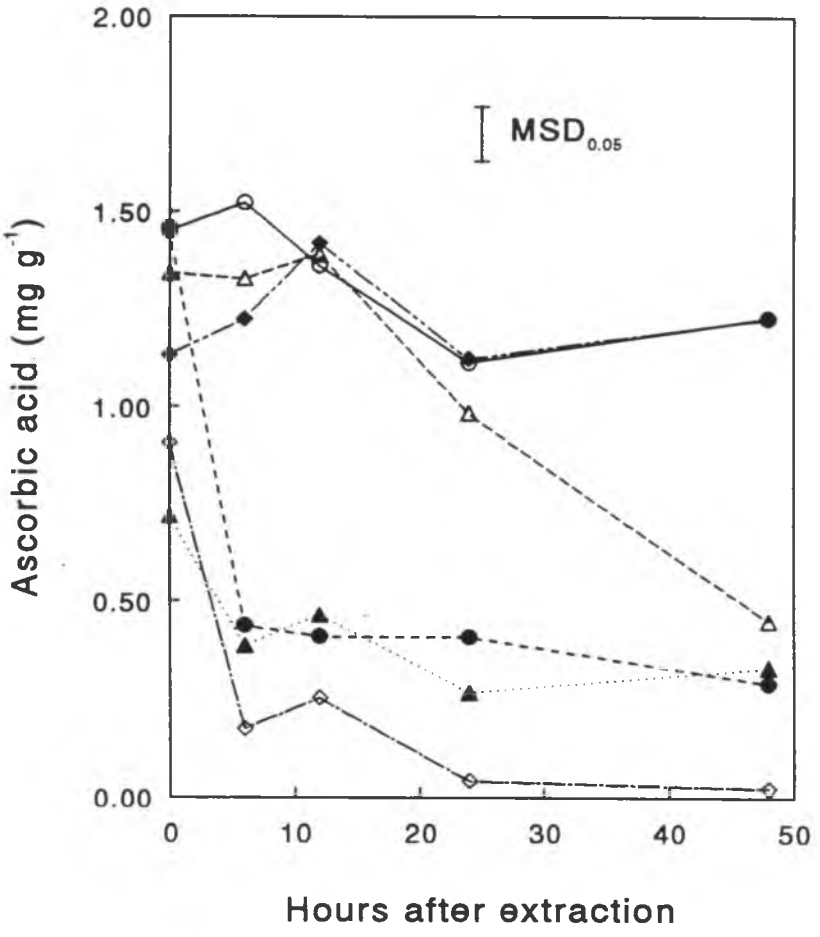

6
4
2
0
MSD0.05
PPO activity (units/ml)
0
6
12
18
24
Hours after extraction

Figure 4.7. Ascorbic acid changes in leaf extracts of *Leucospermum* (o-----o), *Protea* (●-----●), mixture of *Protea* leaf extract with *Leucospermum* at 9:1 ratio (▲-----▲), at 1:9 ratio (◆----◆), at 1:1 ratio (▵-----▵), and mixture of *Protea* leaf extract with water 1:1 (◇----◇). Data were analyzed by Duncan-Waller multiple range test, $MSD_{0.05}$ = mean significant difference ($P<0.05$, n = 6).


2.00
1.50
1.00
0.50
0.00
MSD0.05
Ascorbic acid (mg g-1)
0
10
20
30
40
50
Hours after extraction

extract at 1:9 ratio, ascorbic acid decreased significantly 6 hr after incubation to one third of the initial content then remained constant (Figure 4.7).

There was no significant decline or difference in ascorbic acid content between pure *Leucospermum* leaf extract and the mixture of *P. neriifolia* with *Leucospermum* leaf extract at 1:1 ratio during the first 24 hr of incubation (Figure 4.7). However, 48 hr after extraction, when the mixture became brown, ascorbic acid content had decreased significantly to one-third of the initial value (Figure 4.7).

Leaf pH. The pH of *P. neriifolia* and *Leucospermum* leaf extract, mixture at 1:1 ratio, and *P. neriifolia* leaf extract with water were determined during 24 hr of incubation. The pH of *Leucospermum* leaf extract was low (4.4) and constant (Figure 4.8). The pH of *P. neriifolia* leaf extract was significantly higher (5.2) than *Leucospermum* leaf extract (Figure 4.8) and increased slightly after 6 hours of incubation, but then the pH declined significantly afterwards, to about 4.6, 24 hr after incubation (Figure 4.8). The diluted *P. neriifolia* leaf extract with water had similar pattern of pH changes as a pure *P. neriifolia* leaf extract (Figure 4.8). Mixing *P. neriifolia* leaf extract with *Leucospermum* leaf extract (1:1) significantly lowered the pH of *P. neriifolia* leaf extract to 4.6 (Figure 4.8).

Substrate concentration and inhibitory effect. *Protea* leaf PPO activity increased with an increasing catechol substrate concentration from 1 mM to 60 mM, then declined at higher concentrations (Figure 4.9). Adding a small amount of *Leucospermum* leaf extract to the above reaction solution significantly decreased *Protea* PPO activity (Figure 4.7). When 10 µl of *Leucospermum* leaf extract (protein

Figure 4.8. Changes in pH of leaf extracts of *Leucospermum* (o-----o), *Protea* (●-----●), mixture of *Protea* leaf extract with *Leucospermum* at 1:1 ratio (▵-----▵), and mixture of *Protea* leaf extract with water 1:1 (▲-----▲) incubated for 24 hours. Data were analyzed by Duncan-Waller multiple range test, $MSD_{0.05}$ = mean significant difference ($P<0.05$, $n = 6$).

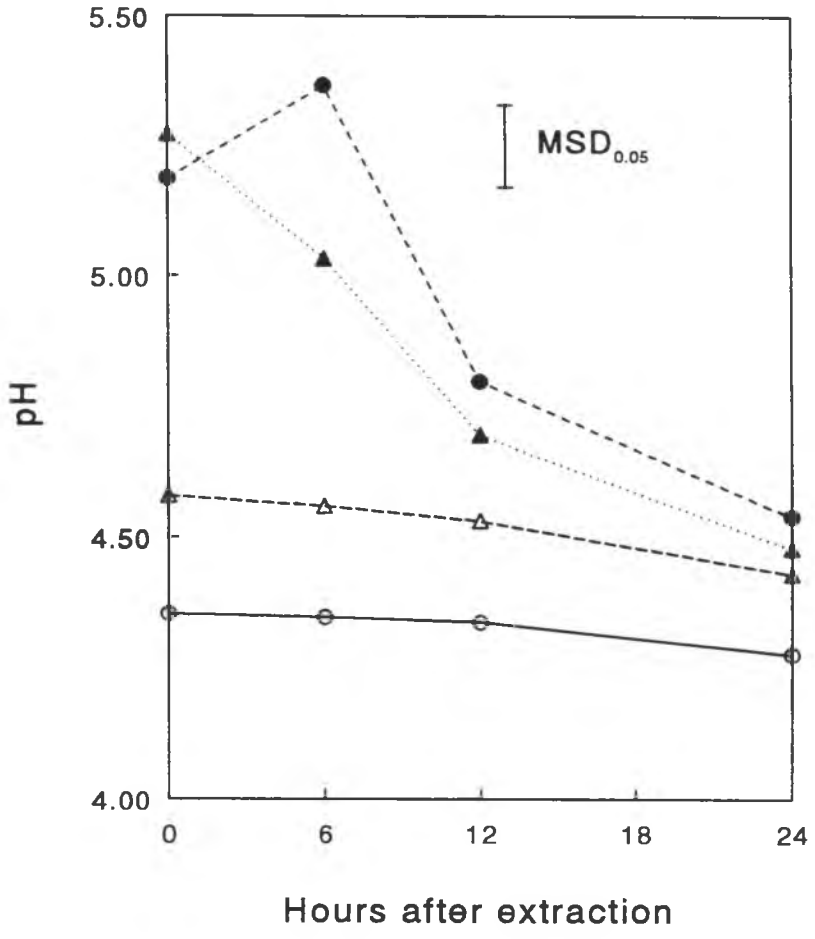
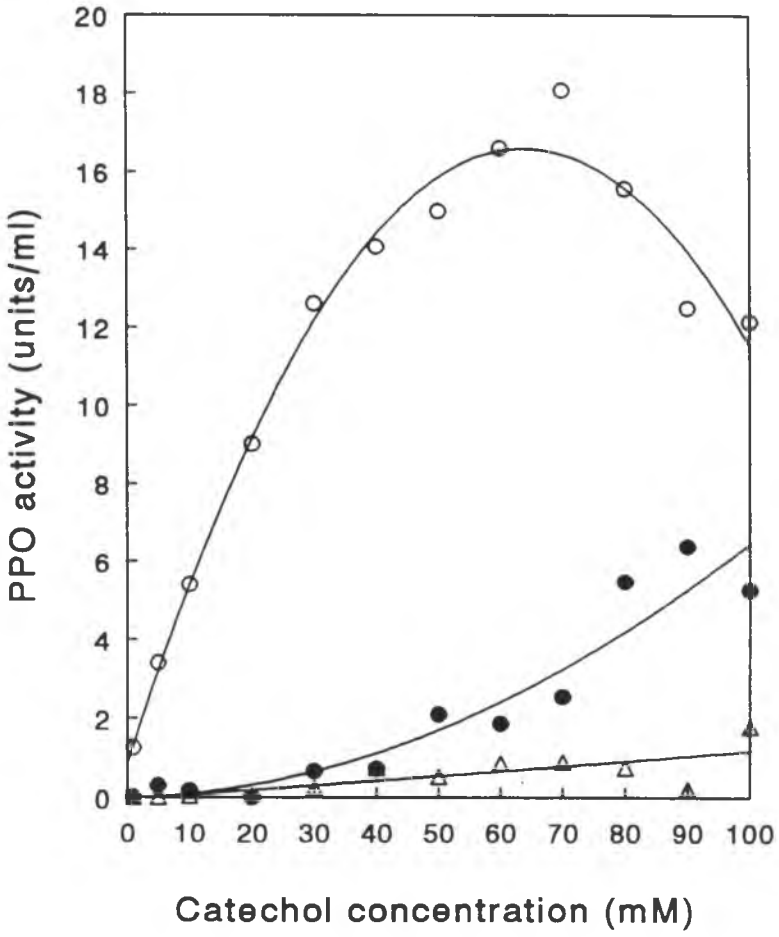

5.50
5.00
4.50
4.00
pH
MSD0.05
0
6
12
18
24
Hours after extraction

Figure 4.9. Polyphenol oxidase activity in *Protea* leaf with increasing substrate (catechol) concentration, and the inhibitory effect of *Leucospermum* on *Protea* PPO activity. PPO activity in *Protea* leaf (o-----o); adding 10 µl of *Leucospermum* leaf extract to *Protea* leaf extract (●-----●); adding 15 µl of *Leucospermum* leaf extract to *Protea* leaf extract (▵-----▵).

Regression analysis:

Treatments	Equations[z]	r^2
Protea extract	$Y = 0.86 + 0.49X - 0.4 \times 10^{-3}X^2$	0.99***
P. ext.+10 µl *L.* ext.	$Y = -1.3\times10^{-3}+0.3\times10^{-3}X+0.06\times10^{-3}X^2$	0.95***
P. ext.+15 µl *L.* ext.	$Y = 0.08 + 0.8 \times 10^{-3}X$,	0.75**

[z]Y = Predicted PPO activity, X = catechol concentration (mM). **, *** significance at 1% and 0.1% level.


PPO activity (units/ml)
20
18
16
14
12
10
8
6
4
2
0
0 10 20 30 40 50 60 70 80 90 100
Catechol concentration (mM)

content 11 μg) was added to the reaction solution, *Protea* PPO activity was barely detectable until substrate concentration was increased to greater than 50 mM. The PPO activity at 50 mM was ca. 2 units ml^{-1}, 1/7 to 1/9 of the control with no added *Leucospermum* leaf extract (Figure 4.9). Increasing the amount of *Leucospermum* leaf extract to 15 μl (protein content 8.19 μg) inhibited *Protea* PPO activity even further (Figure 4.9).

Whole Flower Stem Study. No leaf blackening was found on *Leucospermum* cut flower stem (Table 4.2). However, *Leucospermum* leaves on the stem did senescence. The symptom of the senescence was grayish and dryness of leaves starting from the leaf edge, spreading inward. The days to 50% leaf senescence of *Leucospermum* cut flower was 16.8, 55% longer than Protea's 10.8 days (Table 4.2).

D. Discussion

Enzymatic browning in plant tissue has been studied in many fruits and vegetables during postharvest handling (Kahn, 1975; Jayaraman *et al.*, 1982; Harel *et al*, 1966; Sapis *et al.*, 1983; Prabha and Patwardhan, 1980; Ben-Shalom *et al.* 1977; Jayaraman and Ramanuja, 1987; Omuaru *et al.*, 1990; van Lelyveld and Bower, 1984; Bower and van Lelyveld, 1985; Cutting *et al.,* 1990). Although enzymatic browning of fruits is mainly attributed to the action of PPO (Jayaraman *et al.*, 1982), it is still not clear whether the enzyme, the substrate, or the combination of the two plays the decisive role in the overall phenomenon of browning. In some fruits, for example, polyphenol oxidase activity plays an important role leading to enzymatic browning.

Table 4.2. Comparison of leaf senescence and blackening of *Leucospermum*, *Protea* cut flowers held in 12 hr light (7-9 μmol m^{-2} s^{-1})/12 hr dark conditions.

Genera	Days to 50% leaf senescence[z]	Leaf Blackening
Leucospermum	16.8 a	No
Protea	10.8 b	Yes

[z]Data analyzed by Duncan Waller multiple range test, means followed by the same latter were not significantly different at 5% level, n = 5.

Degree of browning was found to correlate with PPO activity in avocado (Kahn, 1975), grapes (Sapis *et al.*, 1983), and bananas (Jayaraman, *et al.*, 1982). In others, such as some cultivars of avocado (Prabha and Patwardhan, 1980), apples (Harel *et al.*, 1966), the degree of browning was correlated with substrate concentration. A variegated grapevine mutant Bruce's Sport, known to dry to a lighter color than other seedless varieties, shows reduced PPO activity in mature berries (Rathjen and Robinson, 1992). But in most fruits, the browning potential is presumably determined by enzyme activity, substrate concentration and other endogenous substances such as ascorbic acid, pH and natural enzyme inhibitors. For example, in ripening plantain pulp (*Musa paradisiaca*), the increased browning potential observed with ripening has been related with relatively high PPO activity and level, low ascorbic acid content and high total phenolics (Omuaru *et al.*, 1990). During ripening in some banana varieties, a combination of factors involving PPO, ascorbic acid and pH influence the susceptibility of the pulp to browning and no one particular factor can be attributed to exclusively influence browning (Jayaraman and Ramanuja, 1987). The browning potential of olives during fruit development is also determined by enzyme activity and substrate concentration (Ben-Shalom *et al.* 1977).

Protea leaves are highly susceptible to blackening. The browning process has been described in the Literature Review (page 6), and the blackening was the result of higher molecular weight polymerization (Labuza and Schmidl, 1986). In contrast, *Leucospermum* leaves did not turn brown or black under any of the conditions tested, such as mechanical injury (Figure 4.1), senescence in the dark as leaf discs

(Figure 4.2) or attached to the cut flower stem (Table 4.2). PPO activity was very high in *Protea* leaves (14 units) compared to avocado (2.7 units, Van Lelyveld and Bower, 1984), and bananas (6.4 units, Jayaraman *et al.*, 1982), while in *Leucospermum* PPO activity was not detected (Table 4.1). Also, *Protea* leaf had higher level of total phenolic compounds, 4 times higher than *Leucospermum* leaf (Table 4.1). The high PPO activity and high total phenolic compounds in *Protea* leaf may explain why *Protea* leaves were so susceptible to browning and blackening.

Ascorbic acid is a naturally occurring inhibitor of enzymatic browning (Labuza and Schmidl, 1986). It is known to act by reducing the quinones formed during enzymatic oxidation back to phenolic compounds, accompanied by a gradual decrease in PPO activity due to reaction inactivation. Therefore, endogenous level of ascorbic acids could influence the browning potential of plant tissues. A combination of low PPO activity and high ascorbic acid content is found in the least susceptible banana cultivar 'Puttabale' (Jayaraman *et al.*, 1982). However, no significant difference in ascorbic acid level was found between *Protea* and *Leucospermum* leaves (Table 4.1), although its level in *Protea* leaf extract declined significantly after 6 hr of standing while no change was found in *Leucospermum* leaf extract (Figure 4.7).

The natural pH of plant tissue has not so far been indicated as a factor that could influence the browning potential. However, in some banana varieties, the natural pH of the pulp is found to exert some influence on browning (Jayaraman *et al.*, 1982). This influence may relate to PPO activity. It is known that PPO from

different plant sources exhibit different pH optima and the pH optimum of most PPO studied is between pH 5.0 and 7.0 (Labuza and Schmidl, 1986). In some banana varieties, the nearer the pH to 7.0, the higher the susceptibility to browning (Jayaraman *et al.*, 1982). For example, at the pH of 5.2 associated with the relatively highly susceptible 'Pachabale', the PPO exhibited about 57.5 per cent of its activity at the optimum pH of 7.0, while at pH 4.5 to 4.6 associated with the least susceptible varieties, the enzyme showed only 6 to 11 per cent of the activity at the optimal pH. PPO preparations from several sources are reported to be inactivated below pH 4.0 (Thomas and Janave, 1973). The low pH of *Leucospermum* leaf (4.4) compared to high pH in *Protea* leaf (5.5) may also be a factor influencing the blackening potential.

Leucospermum leaf may lack PPO activity, and had an inhibitory effect on *Protea* PPO activity (Figure 4.9). Only small amount of *Leucospermum* leaf extract (10 μl with protein content 11 μg) decreased PPO activity in Protea leaf extract more than 10 fold. However, increasing substrate concentration decreased the effect (Figure 4.9), indicating that the inhibitory effect may be competitive in nature. The effect of PPO inhibition increased as the amount of *Leucospermum* leaf extract increased, suggesting again that *Leucospermum* leaf may contain competitive inhibitors for PPO activity. However, we do not know whether this inhibitor functions *in vivo*. Naturally occurring PPO inhibitors have been detected or isolated from various tissues (Mayer and Harel, 1979). Most of them are low molecular weight oligopeptides which competitively inhibit the enzyme by reacting with the

copper (Mayer and Harel, 1979). Labuza (1989) reported that certain fruit extracts containing proteases, particularly ficin from fig (*Ficus* sp.), inhibit browning in fruit. Anon (1990) also reported that the protease from fig latex, appear to function as browning inhibitors in several food systems. The mode of action of the ficin suggested by the authors is to inactivate the PPO enzyme via proteolysis. However, since the ability of ficin to inhibit browning is unaffected by its heat denaturation (Anon, 1990) or ultrafiltration (McEvily, 1991), McEvily and Lyengar (1992) suggested that other nonenzymatic factors are probably involved in the fig extract inhibition of browning. Based on analytical data for homogeneous preparations, three inhibitors present in the fig extract were found to be analogous 4-substituted resorcinols (McEvily and Lyengar, 1992). These compounds, identified as 2,4-dihydroxydihydrocinnamic acid, 2,4-dihydroxydihydrocinnamoyl putrescine, and *bis*-(2,4-dihydroxydihydrocinnamoyl)-spermidine, are novel, plant secondary metabolites (McEvily and Lyengar, 1992). Two of these are substituted polyamines which also have some anti-senescence and membrane stability effects. The *Leucospermum* leaf extract lowered *Protea* leaf extract's pH (Figure 4.8), delayed the reduction of *Protea* leaf extract's ascorbic acid (Figure 4.7) and reduced *Protea* leaf extract's blackening (Figure 4.5). The nature of the inhibitor is unknown.

The postharvest leaf blackening of *Protea* was influenced by a number of interacting factors. The complicated nature of this process requires an approach that allow definitive further investigation. Comparing the biochemical characteristics of two different species or genera having different susceptibility to enzymatic

browning/blackening, can provide an understanding of the nature of the blackening and probably can lead to a method to prevent blackening. Leaf blackening may be solved not only through postharvest handling, but may also through other measures such as plant breeding, by selecting less susceptible cultivars, or even genetic engineering.

IX. CONCLUSION

Protea postharvest leaf blackening is complicated by many postharvest factors: water stress, darkness, and flower head sink demands. The symptoms occurring under different stress conditions gave different patterns: browning from the midvein of leaves occurred when water stress was present, marginal and spotted leaf blackening was the symptom of leaves held in the dark. In the dark, leaves showed higher CO_2 evolution, leaf exudate with higher ion concentration, and higher soluble polyphenol oxidase activity. Sucrose (2.5% to 5% w/v) provided substrate for leaf respiration, prevented ion leakage, protected PPO latency, and prevented leaf blackening in the dark.

Protea leaf blackening was influenced by flower head sink demand for carbohydrates. The flower head constituted 2/3 of the stem fresh weight and continued to grow, open, respire, and produce nectar after harvest. An average of 9.8 ml nectar with 24% sugar can be collected from a mature flower head. This was ca. 2.4 gm of sugar per flower head. Therefore, the potential sink strength is high, requiring energy and carbohydrate supply for the growth of the flower head and the production of nectar. This carbohydrate source can be the head itself, the stem, the leaf, or the whole shoot. The relationship between flower head sink demand and leaf source supply was shown using ^{14}C-sucrose application to a leaf for 24 hours. More than 50% of radioactivity was found in nectar when nectar production was highest. When the flower head was removed, or a portion of the stem immediately below the flower head was girdled, or flower stems held under bright light condition, or

appropriate amount of sucrose (2.5% to 5%, w/v) was added to the vase solution, the rate of leaf blackening was either delayed or even prevented. These results suggest that leaf carbohydrate depletion in the leaf is the main factor influencing the blackening, and the carbohydrate depletion occurs when flower stems are held in the dark, accelerated by presence of the flower head and nectar production.

Polyphenol oxidase activity, total phenolics content, ascorbic acid content and pH of the plant tissue are the four characters used to study the susceptibility to browning of many fruits. *Leucospermum*, another genus in the same family with *Protea*, did not show leaf blackening under any circumstances. Although it had no significant difference in ascorbic acid content, it had lower total phenolic compounds, lower pH value, and strikingly, no PPO activity. It also showed an inhibitory effect on *Protea* leaf PPO activity. But the nature of the inhibitor was not known.

Based on these studies, the following recommendation can be made regarding to commercial practices:

1. Harvest flowers in the afternoon when there are more carbohydrates in the leaf due to photosynthesis;

2. After harvest, place flower stems in water as soon as possible to reduce water loss;

3. Store flower stems in bright light condition, with light levels higher than leaf light compensation point (PAR > 10 μmol m^{-2} s^{-1});

4. Provide flower stems with flower preservatives containing sugars or with sucrose solution; the recommended concentration for sucrose is in the range of 2.5% to 5% (w/v).

5. Leave as many leaves on flower stem as possible, the more leaves on flower stem, the longer the vase life.

6. Store flowers in proper temperature conditions to reduce excess carbohydrates consumption.

7. Pulsing flower stem with sucrose (20% w/v) overnight before shipping has been suggested by Reid (unpublished).

Further research is needed in the area of plant breeding selection to look for less susceptible lines to blackening.

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